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THE BACTERIOSTATIC AND GERMICIDAL PROPERTIES OF BILE

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. VII

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Synopsis

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Brief review of the literature.
Methods.
The qualitative germicidal action of hepatic duct and gallbladder bile of rabbits, guinea-pigs and dogs.
Notes on the basic qualitative action of hepatic duct and gallbladder bile of the cat, goat, rat, monkey, ox, pig, sheep and man.
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The growth of B. typhosus and Vibrio cholerae in human cystic bile at varying H-ion concentrations.

The problem of the typhoid carrier state, particularly the condition in which the prolonged sojourn of B. typhosus occurs in the gallbladder and the biliary passages, cannot be solved conclusively without giving due consideration to the biliary secretions. Some of the factors which are obviously responsible for the prompt localization of the typhoid bacillus, at least in the gallbladder of certain laboratory animals, have been discussed and illustrated in the preceding papers of this series.

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With the exception of Nichols,[1] and quite recently Beckwith,[2] no investigator interested in the experimental aspect of the carrier state paid sufficient attention to the bile. Nichols claimed that the bile of rabbits and guinea-pigs is strongly alkaline and therefore antiseptic. This conception is rather surprising and contrary to the commonly expressed belief in the literature that bile is a selective stimulating substance favoring the persistence of the typhoid bacillus. However, certain references supply data which confirm the conclusions of Nichols. The following questions, therefore, suggested themselves for investigation: 1. Is the bile of animals and man antiseptic, inhibitive or stimulating? 2. If antiseptic, is the sterilizing effect of bile due to the content of antibodies or due to chemical substances, or both? A study of the phase mentioned under (1) discloses two outstanding facts: First, we are unable to test with the present methods at our disposal the properties of bile in vivo. Second, in vitro experiments with bile must be considered test-tube artefacts. Our experiments in general support the view that bile in vivo is a favorable medium for the typhoid-paratyphoid-group of bacteria.

TABLE 1

BACTERIOLYTIC POWER (?) OF HUMAN BILIARY FISTULA-BILE FOR TYPHOID BACILLI

Case	Treatment	Dosage of Bacilli per C c of Bile	Progress of Sterilization Day					
			1	2	3	5	10	15
L. St. 1917	Daily sodium Bicarbonate Magnesium sulfate and Carlsbader salt	500,000	+	+	+	+	+	+
		50,000	+	+	+	+	+	+
		5,000	+	+	+	+	+	+
		1,000	+	+	+	+	+	+
		100	+	+	+	+	+	+
		50	+	+	+	+	+	+
		25	+	+	+	+	+	+
		10	+	+	+	+	+	+
		5	+	+	+	+	+	+

REVIEW OF THE LITERATURE

Since 1884 nearly 50 authors have tested bacteriologically the properties of gallbladder and common-duct fistula bile of a variety of animals and also of man. The reports can be analyzed only with difficulty, on account of the diversity of methods and bile specimens employed. Some investigators tested fresh bile, others used old heated and sterilized biles in undiluted form, and still others added bile or bile salts in varying amounts to liquid or solid nutritive substratums. Failure to appreciate the various factors which may alter the same bile specimen in reaction or in chemical composition and lead to a change in its properties as a culture medium, has produced an array of contradictory statements. Quantitative experiments have also been considered in a few instances, and biles collected in different localities have been tested with a variety of pathogenic organisms. Conclusions drawn from observations on ox bile have frequently been applied to other animal biles without the least experimental investigation. Based on the available data, let us see how far we are able to answer the question: Is bile antiseptic or not? Here again we meet with difficulties, because four different properties have been ascribed to the bile in vitro, namely, it favors the growth of the common bacteria, except pneumococci and streptococci, or it is indifferent or it can act as an inhibitive or even as a germicidal fluid on the bacterial proliferation. Some experiments have been recorded also, in which the rate of the inhibitive action invariably has led to a complete sterilization of the bile sample. We have observed the four phases in one bile sample and intend to consider the mechanism which produces the various end-results more carefully in the course of the analysis of our data. The findings published deal with the gallbladder bile of oxen, dogs, pigs, man, guinea-pigs and rabbits. Some observations made on fistula bile of rabbits and man are also recorded.

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[1] J. Exper. Med., 1916, 24, p. 497.

[2] Thesis, 1920.

Unquestionably the most detailed studies were made with ox bile. Babes,[3] Dünschman,[4] Fisher,[5] Lagane,[6] Neufeld,[7] Ottolenghi[8] found it to be a growth-favoring fluid for typhoid, coli and other intestinal organisms, even the cholera vibrio. Bernabei[9] (not Corrado), Braun,[10] Charrin and Rogers,[11] Flu,[12] Gley and Lambling,[13] Leubuscher,[14] Nichols,[1] Vetrano,[15] Violle[16] report ox bile to be

[3] Berl. klin. Wchnschr., 1899, 36, p. 361.
[4] Ann. de l'Inst. Pasteur, 1909, 23, p. 29.
[5] Thesis, Bonn, 1894.
[6] Compt. rend. Soc. de biol., 1912, 73, p. 242.
[7] Neufeld, O., and Handel, O.: Arb. a. d. k. Gsndhtsamte, 1908, 28, p. 572.
[8] Centralbl. f. Bakteriol., O. I, 1911, 58, p. 120.
[9] Ibid., 1892, 12, p. 696; Atti dell Accad. Med. de Roma, 1890-91.
[10] Arch. d. Sc. biol., St. Petersburg, 1901, 8, p. 158.
[11] Compt. rend. Soc. de Biol., 1886, 3, p. 425.
[12] Geneesk. Tijdschr. v. Nederl. Indie, 1918, 58, p. 67.
[13] Rev. Biologique du Nord de la France, 1888, 1, p. 28.
[14] Ztschr. f. klin. Med., 1890, 17, p. 472.
[15] Centrlbl. f. Bakteriol. I, O., 1909, 52, p. 275.
[16] Ann. de l'Inst. Pasteur, 1912, 26, p. 381.

indifferent. Copeman and Winston,[17] Ehret and Stolz,[18] Ecker,[19] Fornet[20] and Jordan,[21] Obst,[22] Pies,[23] Tonney, Caldwell and Griffen[24] proved, however, conclusively, that this secretion is a poor culture medium, and when carefully tested exhibits regularly at least transitory inhibitive properties. Dog bile was found to be indifferent or slightly stimulating by Létienne[25] and Miyake,[26] while Ehret and Stolz[18] and Toida[27] noticed inhibition of bacterial growth. According to Leubuscher,[14] B. typhosus and B. coli grew well in pig's gallbladder bile.

Fresh human bladder bile can according to Babes,[8] Fränkel and Krause,[28] Fütterer,[29] Hirokowa,[30] Kramer,[31] Leubuscher,[14] Mieczowski,[32] Nichols,[1] Pies,[23] Toida[27] and others act as a good culture medium. Occasionally samples are found which inhibit strongly the representative organism of the colon-typhoid group (Pies,[23] Hirokowa,[30] Toida,[27] Copeman and Winston,[17] Ehret and Stolz[18] and Twort and Pastia.[33]

Guinea-pig's bile added to gelatine was found to be inhibitive by Copeman and Winston. Fresh alkaline fistulous bile of these animals is, according to Nichols,[1] strongly antiseptic. Vallardi and Bezzola[34] state that in guinea-pigs the resistance of the intestines to B. typhosus is not due to the pancreatic or biliary secretion.

Lange and Roos,[35] Rolly and Liebermeister,[36] Talma[37] and Nichols[38] reported the gallbladder bile of some rabbits as slightly or strongly inhibitive for B. typhosus, B. coli, etc. Vieillard-Baron,[39] Vincent[39] and Violle[16] found the antiseptic properties to be feeble or absent. Violle (p. 398), expresses himself on this subject as follows: "Il en est de même avec la bile de lapin ensemencée avec les bacilles typhiques, coli et le vibrion cholerique. Bien mieux, ce sera un milieu de predilection pour ces elements bacteriens, qui s'y developperont avec une rapidité beaucoup plus grande que dans toute autre substance nutritive." Nichols[1] tested the bactericidal properties of hepatic duct bile and found it strongly antiseptic for B. typhosus, B. paratyphosus A, B. coli and B. dysenteriae. The antiseptic action is, in his opinion, largely due to its alkalinity, which also explains his observation that Vibrio cholerae, an alkalophilic micro-organism, grew well in fresh rabbit bile. Aside from the carefully studied selective action of bile and bile salts on pneumococci, numerous reports indicate that one and the same sample may be antiseptic for one group and indifferent to another. Equally important and subject to further investigation is the rather superficially studied time rate of the inhibitive action or the velocity of the sterilizing process in various biles. Ehret and Stolz,[18] Fornet,[20] Pies,[23] and Ecker [19] supplied in this connection interesting data, which indicate that a small number of organisms is inhibited to a relatively greater degree than the large inoculum which is ordinarily chosen by the laboratory worker.

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[17] J. Physiol., 1889, 10, p. 213.

[18] Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 350; 7, p. 372.

[19] J. Infect. Dis., 1918, 22, p. 95.

[20] Arch. f. Hyg., 1907, 60, p. 134.

[21] J. Infect. Dis., 1913, 12, p. 326.

[22] J. Bacteriol., 1916, 1, p. 73.

[23] Arch. f. Hyg., 1907, 62, p. 107.

[24] J. Infect. Dis., 1916, 18, p. 239.

[25] Arch. de méd. expér. et d'anat. path., 1891, 1, p. 761.

[26] Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 479.

[27] Arch. f. klin. Chir., 1913-14, 103, p. 407.

[28] Ztschr. f. Hyg. u. Infectionskrank., 1889, 32, p. 97.

[29] München. med. Wchnschr., 1888, 35, p. 315.

[30] Centralbl. f. Bakteriol., O. I, 1909-10, 53, p. 12.

[31] J. Exper. Med., 1907, 9, p. 319.

[32] Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 307.

[33] Compt. rend. Soc. de biol., 1911, 71, p. 13.

[34] Abstr. in Off. internat. d'Hyg. Publique, 1918, 10, p. 921.

[35] Arb. a. d. Gsndhtsamte, 1915, 50, p. 57.

[36] Deutsch. Arch. f. klin. Med., 1905, 83, p. 413.

[37] Ztschr. f. klin. Med., 1901, 42, p. 354.

[38] Jour. Am. Med. Assn., 1917, 68, p. 958.

[39] Vieillard-Barron: Thesis No. 1038, Lyon, 1895. Ann. de l'Inst. Pasteur, 1908, 22, p. 341; Compt. rend. Soc. biol., 1916, 79, p. 580. Vincent, M. H., and Marbé: Compt. rend. Soc. biol., 1917, 80, p. 587. Vincent, M. H., and Fauré-Fremiet, E.: Compt. rend. Soc. biol., 1917, 80, p. 589. Vincent, Marbé and Muratel: Compt. rend. Soc. biol., 1917, 80, p. 675.

From this brief analysis it can be concluded that the results obtained by various workers with bile from the same type of animal and with the same bacterium are more or less at variance, but the majority of writers obtained results which indicate that bile in vitro may be more or less inhibitive, even slightly germicidal. Only Talma,[37] Violle [16] and Hailer and Ungermann [40] conducted in vivo experiments on the bactericidal properties of rabbit bile by the direct inoculation of B. typhosus into the gallbladder content of these animals. For example, Hailer and Ungermann noted regularly a development of the introduced bacilli in the gallbladder bile, irrespective of the number chosen, and 93% of their animals were found infected up to the 31st day after the inoculation. Less uniform were the results obtained by Talma,[37] who also injected typhoid and colon bacilli into the gallbladder of rabbits. The following points in his conclusions are of some interest: Rabbit bile contains a substance which inhibits most of the typhoid and colon bacilli. The bactericidal property is probably the result of chemical action and the antiseptic action of rabbit bile varies in individual animals and at different time periods. If we accept, therefore, the conclusions, which are well supported by the recent careful studies of Jordan [41] and Ecker,[19] that bile is inhibitive even germicidal to a certain degree, we immediately have to answer the question: Is this antiseptic effect the result of (1) antibodies, or (2) chemical substances?

The old Italian and French medical literature deals extensively with the antitoxic properties and the virulence enhancing or depressing effect of the bile in general. The reader will find a complete review of this phase of the problem in a recent summary of Posselt.[42] On the other hand, definite statements with regard to the presence of specific antibodies in the bile are few and contradictory. Vincent [39] and his associates report on the demonstration of complement-fixing antibodies in a small percentage of rabbits immunized against B. typhosus, but no statement is made that the bactericidal properties of normal bile must be ascribed to such antibodies.

Most of the investigators conclude that the bacteriostatic and germicidal properties are the result of a chemical or of a physical action. In this connection we have to distinguish between an immediate action of the inhibitive substances on the proliferation of the inoculated bacteria and an indirect effect on the fermentative function of the micro-organisms (Roger [43]). The chemical properties and constituents of the bile, which are supposed to exert an inhibitive action on microbes are the reaction, the bile acids and their salts, the biliary lecithins and cholesterol, the biliary mucus, the lack of nutritive material, the surface tension and the photodynamic action of bilirubin.

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[40] Arb. a. d. k. Gsndhsamte, 1914, 47, p. 450.

[41] J. Infect. Dis., 1913, 12, p. 326.

[42] Ergebn. d. allg. Pathologie, 1915, 17, p. 783.

[43] Presse méd., 1913, 19, p. 137; Arch. de med. exper. et d'anat. path., 1913, 25, p. 430.

In 1886 Charrin and Rogers[11] and in 1917 Nichols[1] suspected the reaction of the bile of oxen, rabbits and guinea-pigs to be largely responsible for their germicidal properties on typhoid, dysentery and paratyphoid bacilli. Their findings are in part supported by the observations of Sellards[44] made on pneumococci. The influence of dehydrated ox bile or a mixture of purified bile salts on bacteria has been studied with variant results by a number of workers. Sodium taurocholate (1-10% solutions) was found by Dünschman,[4] Mieczkowski,[32] Mosse[45] and Nicolle and Adil Bey[46] to be either stimulating or indifferent when added to solid or liquid culture mediums, while Bufalini,[47] Bunge,[48] Charrin and Rogers,[11] Lindberger,[49] Limbourg,[50] Leubuscher,[14] Löhlein,[51] Mac Conkey,[52] Maly and Emmerich[53] and Meyerstein,[54] report the free acids or their salts to be decidedly antiseptic. Sodium glycocholate was, in the experiments of Dünschman[4] and of Meyerstein[54] markedly inhibitive. Bassenge[55] considered the biliary lecithin to be a substance of considerable antiseptic power for B. typhosus. His observations that a 1% emulsion kills B. typhosus in from 30 to 60 minutes is not supported by the statements of Charrin and Rogers,[11] who found even a 10% solution to be innocuous. The opinion advanced by Daniel-Brunet and Rolland,[56] that 1% solution of cholesterol may check the proliferation of B. typhosus has recently been confirmed by Manfredi.[57] G. Mayer[58] found the biliary mucin to be inhibitive for diphtheria bacilli, but inactive for B. paratyphoid B, B. coli and B. typhosus. The lack of nutritive substances, particularly in fistular bile, perhaps the absence of vitamines, as suggested from the observations of Pies,[23] Meyerstein[54] and others, are factors which deserve a more thorough investigation in the future. Decidedly novel and of considerable importance are, in our opinion, the recent suggestions offered by Larson[59] and associates, who ascribed the lytic effect of bile on pneumococci to be the result of the well-known low surface tension of this body fluid. The cytolytic action of bilirubin was found to be slight by Charrin and Rogers,[11] while Sellards[44] considered the photodynamic properties of the biliary pigments to be quite marked. Under the conditions prevailing in our experiments the action of these substances was negligible.

It has not been our purpose to investigate all the questions suggested by the review of the literature, but rather to verify some of the published facts bearing on the carrier state and to determine by qualitative and quantitative tests on fresh and old bile samples of a large number of animals the rate of the germicidal action. Experiments have also been conducted to explain the factors responsible for the antiseptic effect, whether biologic or chemical.

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[44] Jour. Am. Med. Assn., 1918, 71, p. 1301.

[45] Ztschr. f. klin. Med., 1889, 36, p. 527; Ztschr. f. Hyg. u. Infektionskrankh., 1900, 34, p. 454.

[46] Ann. de l'Inst. Pasteur, 1907, 21, p. 20.

[47] Bufalini, G.: Boll. d. Soc. tra i cult d. sc. med. in Siena, 1884, 2, p. 142.

[48] Textbook of Physiol. and Path. Chem., 1902, p. 180.

[49] Upsala Läkaref. Förh., 1884, 19, p. 467.

[50] Ztschr. f. Physiol. Chem., 1889, 13, Nos. 1 and 2, p. 196.

[51] Klin. Monatsbl. f. Augsnh., 1908, 46, p. 552.

[52] J. Hygiene, 1908, 8, p. 322.

[53] Sitzungsberichte d. K. Akademie d. Wissenschaften, 1883, 87, p.3.

[54] Centralbl. f. Bakteriol., O., I, 1907, 44, p. 434.

[55] Deutsch. med. Wchnschr., 1908, 34, p. 139.

[56] Compt. rend. Soc. de biol., 1911, 171, p. 298.

[57] Riforma med., 1917, 35, p. 849.

[58] Centralbl. f. Bakteriol., 1899, 25, p. 826.

[59] J. Infect. Dis., 1919, 25, p. 45.

METHOD OF STUDY

In the preceding paper of this series, we have described in detail the technic applied in keeping and collecting specimens of bile.

In the qualitative tests, immediately after titration, definite amounts of bile, usually 1 c c, were pipetted into cotton stoppered, serologic vials (7.5 x 2 cm.). For the quantitative tests triplicate sets of 5 c c of hepatic duct bile and 1 to 2 c c of cystic bile were aseptically distributed in pyrex tubes (100 x 12 mm.) with or without paraffin oil. One set of tubes was used for the determination of the changes in the H-ion concentration, a second for the same procedure after inoculation with bacteria and a third for plating.

When we began our tests a few years ago, we inoculated the bile samples with one loopful (2 mg. loop, 22 gauge wire made on a No. 2 Czaplewski standard rod) of a 24-hour peptic digest broth culture averaging 800,000 organisms for B. typhosus. In later experiments 1 loopful of a 1:1,000 or a 1:10,000 dilution of the same broth culture, approximating from 1,000 to 100 organisms, was used. The seeded tubes were always incubated at 37 C. The progress of the growth or the degree of the sterilization was followed by transferring at regular intervals of 2 hours 1 loopful of bile on to agar slants. It is self-explanatory that the proper inoculation of the bile samples was controlled by streaking an agar slant immediately on seeding of the tubes. Subcultures were made on peptic digest agar P_H^+ 7.4.

For the quantitative tests, 5 c c of bile were inoculated with 0.1 c c of a 24-hour broth culture diluted with distilled water, 1:10,000 or 1:100,000. These dilutions on the average approximated 300 to 500 organisms per 0.1 c c. At varying time intervals 0.1 c c amounts of seeded bile were plated either directly or in progressive saline dilutions. Invariably the determinations of the H-ion concentrations were made at the same time. In the quantitative tests the tubes were incubated in an open water bath or in a desiccator over $CaCl_2$ in an incubator.

A modification of the Buxton[60] method as applied to the determination of the bactericidal properties of serum was also used in some of our quantitative experiments. The procedure was as follows: A 24-hour agar slant culture was washed off with distilled water and standardized to 500,000,000 organisms per c c; 0.1 c c of the dilutions ranging from 1:10 to 1:1,000,000 were transferred into 1 c c lots of bile or serum. After an incubation of 5, 12, 24, etc., hours loop transfers were made on agar slants.

Suitable controls for each experiment, whether noted or not, were treated in exactly the same manner as the biles. The controls were both peptic-digest

[60] J. M. Res., 1905, 13, pp. 305 and 431.

broth P_H^+ 7.4, 0.01% and Witte's peptone solution P_H^+ 7.4, and according to the type of the experiment either distilled water or 0.85% saline solution.

In the quantative test, salt-free buffered broth, according to Dernby and Avery,[61] with hydrogen-ion concentration corresponding to the initial reaction of the biles, namely P_H^+ 7.6 and P_H^+ 8.6, was chosen. Some tubes of this medium were stratified with oil. We failed to note an inhibitive influence of this substance on the growth of the bacteria employed in our experiments.

Representative cultures of B. typhosus (K. and C.), B. paratyphosus A and B, B. dysenteriae, Staphylococcus and other cultures, were chosen which grew better in an alkaline than in an acid medium. In order to check the behavior of the bacteria in a general manner we selected the alkalophilic Vibrio cholerae.

An attempt was made to adapt the B. typhosus to an environment of bile by successive passage through ox bile, but this method was discontinued because no difference in adaptability could be noted. It is generally known that B. typhosus inoculated directly into the gallbladder grows luxuriantly. When such infected bile samples were used for the seeding of hepatic duct bile, it gave no evidence of having acquired increased resistance by its previous sojourn in the gallbladder.

In recording the results of growth on the slants the colonies were counted if they did not exceed 100, otherwise they were noted as positive. The plates prepared in the quantitative investigations were counted after 36 to 48 hours' incubation.

Our tests are divided into two groups, the qualitative and the quantitative. Having established the existence of an antiseptic action of certain bile specimens, we investigated the factors responsible for this property, dealing in the first place quantitatively with the question of bacteriolysins and immune bodies, and secondly, with the chemical aspect of the germicidal substances.

THE QUALITATIVE GERMICIDAL ACTION OF HEPATIC DUCT AND GALLBLADDER BILE OF RABBITS, GUINEA-PIGS DOGS, CATS, GOATS, RATS, MONKEY, OX, PIG, SHEEP AND MAN

Irrespective of initial H-ion concentration of the hepatic duct bile specimen derived from 45 rabbits and 10 guinea-pigs, the various strains of B. typhosus were unable to remain viable for more than 96 hours, if 1 c c lots were used for the tests 5 to 8 hours after collection. On the other hand, gallbladder bile of the same animals that was seeded in the same manner constantly gave positive subcultures for more than 10 days. Hepatic duct bile of rabbits therefore differs fundamentally with regard to its germicidal properties from true gallbladder biles.

The diet apparently influences in a slight degree the sterilizing effect of hepatic duct bile. Base-forming diets, for example, hay, oats, and carrots or cabbage produce a bile which destroys the ordinary stock typhoid strains (K and C) in from 48 to 196 hours. Another strain, more readily adapted to environmental influences, remained viable for periods exceeding 72 to 156 hours. Acid-forming diets, oats and bread, prolonged occasionally the viability for several days. Heating of the hepatic bile specimens for from one-half to one hour at 56 C. shortened the period of disinfection. Slight differences in the rate of action were apparent when 2 c c or 4 c c samples were seeded instead of 1 c c specimens. The rate of lethal action was extended for from 2 to 5 days. With the exception of strain K, a small number of typhoid bacilli (100-200 bacteria) was more rapidly destroyed than the usual number of several thousand. This observation is quite in accordance with the findings of Ehret and Stolz,[18] of Fornet [20] and of Pies.[23]

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[61] J. Exper. M., 1918, 28, p. 345.

A selective action of the germicidal substances of rabbit hepatic duct bile on various bacteria was also noted. It is most unfortunate that technical difficulties make it impossible to collect a sufficient amount of pure cystic bile for similar extended tests, but it is evident from the few data collected that the selective action is probably entirely absent or at least not as striking as is the case for hepatic duct bile. Paratyphoid bacilli behave similarly to B. typhosus; B. coli is somewhat more resistant; while Vibrio cholerae and Staphylococcus aureus must be looked on as somewhat better adapted species for growth and resistance in fistula bile of rabbits kept on mixed or on acid-forming diets. It should, however, be emphasized that even these organisms cannot lead to an indefinite existence on this type of bile, because subcultures can rarely be found positive after 10 days of incubation. The composition of the bile as a result of diet influences the selective action of Staphylococcus and Vibrio cholerae to slightly more marked degree, and variant results are more frequently encountered than with B. typhosus. The dysentery bacilli are considerably less resistant to the germicidal action; they rarely survive, even if inoculated in large doses. The only exception was the observation made on a bile specimen collected from fasting animals in which the bacilli lived for a period of 36 hours.

Guinea-pig hepatic duct bile exhibits greater disinfecting properties against the same bacteria than rabbit duct bile. The time rate of lethal action is shortened to 36 and 48 hours and even for Vibrio cholerae it never exceeds 5 days. Staphylococcus occasionally remains viable, even when transferred to this bile in rather small numbers. Otherwise the action seems to follow the same course as in the case of the hepatic duct bile of rabbits. It is usually impossible to obtain from guinea-pigs operated on for a common duct fistula sufficient cystic bile for a bacteriologic study. We therefore tested a large series of gallbladder biles collected from various necropsy examinations on animals submitted to anaphylaxis tests or those which succumbed to suffocation in a shipment. Most of the samples stood at room temperature for 24 to 48 hours, while the set of specimens collected from suffocated animals was inoculated and tested immediately.

Gallbladder bile specimens which appeared physically normal destroyed typhoid bacilli in 24 to 28 hours, that is to say, in a somewhat shorter time interval than hepatic duct bile. Three specimens which appeared colorless and physically changed acted more slowly in from 48-96 hours. The addition of blood prolonged the time rate of lethal action for over 240 hours. Fresh bile of suffocated guinea-pigs was not bactericidal for B. typhosus during the observation period of 400 days, but destroyed Vibrio cholera in 216 and 312 days, respectively. Old gallbladder bile of guinea-pigs may be antiseptic for cholera vibrios in a somewhat shorter time interval than hepatic duct bile. A few biles inoculated simultaneously while pipetting the samples into test tubes, and incubated at once showed a viability for B. typhosus of from 5 to 6 days.

In conclusion, we can state that our studies with bile specimens of rabbits and guinea-pigs have demonstrated that hepatic duct bile collected from rabbits and guinea-pigs in open tubes is antiseptic for B. typhosus, B. paratyphosus A, B. dysenteriae, B. coli, Vibrio cholerae, and Staphylococcus aureus. Pure, fresh cystic bile of rabbits and of suffocated guinea-pigs either lacks this property entirely or shows it in a less marked degree. The action is selective and apparently influenced by the diet. But we desire to emphasize the fact that the time rate of lethal action has never been less than 48 hours for the B. typhosus in hepatic bile of rabbits or 36 hours for that of guinea-pigs. Our observations made on a large series of animals differ therefore materially from those reported by Nichols,[1] who claims that with the exception of Vibrio cholerae one loopful of B. typhosus, B. coli, etc., was regularly killed by duct bile in 24 hours. He failed to state in his first paper whether this germicidal action is only to be attributed to the common duct fistula bile or to the cystic bile or to both. After we had called his attention to our results, he corrected this impression by stating that "the effect (antiseptic) was first noted with bile from a common duct fistula. It is not so definite, especially in the case of the rabbit, with bile from the gall-bladder, which is less alkaline and contains more solids than bile directly from the liver." [38] In subsequent paragraphs we shall explain the factors responsible for the differences between Nichols' and our observations, and discuss in detail his conception that the reaction of the bile is responsible for the germicidal action.

Pure gallbladder bile of rabbits in vitro has no antiseptic properties. It may be inhibitive to a slight degree or a poorer medium than broth and may even destroy after prolonged incubation (9 to 20 days) at 37 C. a small number of typhoid bacilli, but it certainly exhibits a different action than does the hepatic duct bile. As the conclusions of Nichols are based on the observations with the latter, his arguments that the bile is an important factor in the development of typhoid carriers are materially deprived of support by our own data, a point which will be discussed from various other angles in the course of this study.

The same differences in the sterilizing properties of cystic and hepatic duct bile as discussed for the rabbit and guinea-pig are also present in the dog. Most of the bacteria remained viable in the gallbladder bile for periods extending over 10 days. On the other hand, 4 of the 7 dogs which had gallbladder biles acting indifferently on bacteria, produced antiseptic fistula biles. Typhoid bacilli inoculated in large numbers rarely survived an incubation period of 120 hours. The colon bacillus and staphylococcus resist an exposure for more than 240 days. Heating of the bile to 56 C. for 30 minutes has no influence on the antiseptic properties. Three other dogs furnished hepatic biles which were bactericidal for dysentery bacilli and in one instance also for a small number of typhoid bacilli. The dogs were all kept on a meat and bread diet and had received inoculations of sodium taurocholate for reasons already explained in the preceding paper of this series. We are quite aware of the fact that the treatment with bile salts may have produced an abnormal hepatic bile, and that we were actually dealing in these experiments with test tube artefacts. This is probably true to a certain extent, but on further analysis these observations offer some explanation for the antiseptic properties of bile in general, namely, sodium taurocholate in an alkaline solution is destructive for B. typhosus and other organisms. Proper attention must be given to these dog experiments in interpreting the factors responsible for the bacteriostatic and antiseptic properties of bile. Natural gallbladder bile of dogs has bacteriostatic properties and may be considered a fair medium for the development of B. typhosus, B. coli, etc. According to Toida [27] they may remain viable for from 40 to 70 days.

The differences noted in the germicidal behavior of hepatic duct and cystic bile of rabbits, guinea-pigs and dogs suggested some experiments on other animals. With the same technic we failed to detect in cats, goats, rats and monkeys, any bacteriostatic or antiseptic action of hepatic duct bile. At least qualitatively, the liver bile acts similarly to the gallbladder bile. Fresh unheated gallbladder bile of oxen, pigs and sheep collected in December, retained large numbers of viable typhoid bacilli for more than 10 days. This crude method of testing the properties of a bile sample gave the impression that the bile of these animals in comparison with the hepatic duct bile of rabbits, guinea-pigs and dogs is an indifferent or even a good medium for the growth of B. typhosus. Quantitative tests to be described later gave us a better insight into the bacteriologic properties of the bile of these animals and contributed also to the understanding of the factors and the mechanism of the striking lethal action of certain hepatic bile samples.

The few tests conducted with human bile specimens deserve brief consideration. It was not our intention to verify the carefully conducted experiments of Fränkel and Krause,[28] Hirokawa,[30] Toida [27] and others, but we were fortunate in having access to biliary secretions which were collected intra vitam, and to a case of biliary fistula, on which we were enabled to study the influence of an alkaline therapy on the germicidal action of bile.

The gallbladder specimens were obtained either from cholecystectomized bladders or by aspiration at laparotomy. The samples were sterile and were primarily used for the determination of the H-ion concentration. Typhoid bacilli grew well in these secretions and remained viable for more than 10 days; in the only sample tested with the Vibrio cholerae complete sterilization took place on the eighth day. This observation, which confirms that of Toida and which may have some bearing on the cholera-carrier problem, deserves further and more extended investigation.

The hepatic duct bile for our alkaline therapy experiment was collected from a man, 55 years of age, who developed subsequent to cholecystectomy and kinking of the cystic duct, a postoperative fistula. The bile specimens were on several occasions infected with B. coli aerogenes. The patient felt completely comfortable only when taking daily Carlsbader salt; the secretion from the fistula was considerably more mucoid and the jaundice more marked during the few days we excluded this medicament. For a period of several weeks daily doses of 6 gm. of sodium bicarbonate were administered; to this was added magnesium sulfate or Carlsbader salt. We have already discussed in the preceding paper the fact that only slight changes in the reaction were noticeable and that the bile was nearly always neutral to phenolphthalein or 2.2 alkaline to lacmoid with a P_H^+ of 8.0. This bile never exhibited, when repeatedly tested, any germicidal properties, as is shown in table 1. Even such small numbers as 5 to 10 typhoid bacilli grew freely in this medium and remained viable in large numbers for over 15 days. Human fistula bile collected from an intensively alkalinized patient is not antiseptic in test-tube experiments, and it appears to us most unlikely that this property is present in the body. Our observations on rabbits, to be detailed, negative such an assumption.

Qualitative tests with hepatic duct bile collected in test tubes exposed to air from rabbits, guinea-pigs, dogs, cats, monkeys, goats and man, as well as gallbladder biles of the same animals in addition to that of oxen, sheep and pigs, have demonstrated that with the exception of guinea-pig bile, no true germicidal effect was demonstrable in the first 24 hours after the inoculation. The time rate of lethal action varied between 36 and 240 hours for fistula bile of rabbits and dogs, and for gallbladder biles exceeded many times the longest period noted for the hepatic duct biles. There is a significant selective action on B. dysenteriae in comparison to Vibrio cholerae, but one is unable to state whether this is similar to the one commonly observed with chemical or biologic bactericides, or whether it is due to an entirely new property. It was noted that in hepatic duct bile of rabbits an appreciable increase of organisms occurred between the 5th and 12th hour after the inoculation of the specimen with a few bacteria, and yet transplants from such tubes at the 24th and 48th hour gave complete sterility. Quantitative tests alone can explain these differences and possibly assist in the understanding of the cycle of events which take place in the bile removed from the body and placed in an open test tube.

THE PROGRESS OF STERILIZATION OF NATURALLY INFECTED GALLBLADDER BILE OF RABBITS, GUINEA-PIGS, MONKEYS AND MAN IN VITRO

In the preceding paper we have called attention to the low H-ion concentration of gallbladder carrier bile of rabbits, and we explained this observation by the fact that the gallbladder wall is seriously damaged and that the infected secretion is mainly hepatic duct bile. A study of the noninfected hepatic duct bile was therefore essential to a clear understanding of the relationship of bile to the carrier state, a phase of the problem which had been qualitatively dealt with in the preceding paragraphs. For this purpose a large series of gallbladder biles collected from rabbits in various stages of infection was tested qualitatively over periods extending to two months. A few unselected observations are shown in table 2.

Nearly one-half of the rabbit gallbladder biles infected for varying time intervals became sterile in from 4 to 10 days. According to the available notes, these biles were limpid, light green and contained macroscopically no pus or carbonate débris. The number of viable typhoid bacilli determined by plating frequently exceeded one million per c c. The other half of the carrier biles were all more or less purulent or distinctly viscid, some forming, on standing, a heavy sediment of greenish, sand-like carbonate débris. In these samples B. typhosus or B. paratyphosus remained viable for more than 30 days, irrespective of the agglutinin content. The physical consistency of the bile, and not the duration of the carrier state, governed the persistence of viable organisms. In view of these results it is evident that removal of the bile from the gallbladder and its exposure to air may have produced antiseptic substances. In all probability these did not, and would not, have developed in the same time interval in the gallbladder in vivo. Progressive decrease in the H-ion concentration in pus-free bile specimens is probably one of the factors responsible for this action. In purulent carrier biles in vitro the greater viability of B. typhosus is probably explained by an absence of a decrease in the H-ion concentration as a result of constant CO_2 formation by the pus cells in the sediment, or later the formation of lactic acid as a result of a destruction of cells on standing. It is of interest in this connection to recall that in the course of these tests we became convinced that removal from the animal body is conducive to alteration in the physical and chemical composition of the bile, and that exposure to air produces an unsuitable medium for B. typhosus. It also became apparent that it is obviously wrong to draw conclusions relative to the action of rabbit bile on B. typhosus when the secretion is exposed to air. The time rate of lethal action was probably a variant factor on account of the different composition and content of bile salts.

The observations made on rabbit bile were confirmed by similar tests with guinea-pig and monkey bile specimens. Dog biles were

TABLE 2

Progress of Sterilization of Naturally Infected Gallbladder Biles of Rabbits, Guinea-Pigs, Monkeys and Man

Rabbit	Method of Infection and When Killed	Agglutination Reactions		Number of B. Typhosus (Polyvalent) per C c of Bile	Incubated at 37 C. Found Sterile on
		Serum	Bile		
1593	Intravenous, killed 120 hours after injection	40,000	100	50,000 per 8 c c no pus	19th day
1595	Intravenous, killed 10 minutes after injection	1:10++	1:10++	1334 per 0.5 c c no pus	10th day
1609	Carrier, 48 days' duration	1:10,000	<1:10	>5,000,000 per c c pus	21st day
1630	Carrier, 117 days' duration	1:8000	1:10++	>5,000,000 per c c pus	>20th day
1591	Intravenous, 96 hours after injection, killed	1:60,000	1:20+++	1,420,000 per c c no pus	21st day
1715	Intravenous, 48 hours after injection, killed	1:10+++	<1:10	140,000,000 per c c	>20 days
1720	Intravenous, 10 minutes after injection, killed	<1:10	<1:10	30 per c c	>20 days
800	Carrier for 84 days, chloroformed	1:1000	....	About 50,000 per c c	4 days
805	Carrier for 84 days, chloroformed	1:4000	....	>1,000,000 per c c	4 days
806	Carrier for 83 days, chloroformed	1:6000	....	>1,000,000 per c c	4 days
912	Carrier for 64 days, died	1:1000	....	>1,000,000 per c c	>60 days
926	Paratyphoid B carrier 120 days, died	1:4000	....	>1,000,000 per c c	>20 days
954	Typhoid carrier for 17 days, died	1:80,000	....	>1,000,000 per c c	>20 days
972	Typhoid carrier for 10 days, chloroformed	1:400	....	>1,000,000 per c c	5th day
963	Typhoid carrier for 272 days	1:200	1:10++	12,000,000 per c c	9th day
1028	Typhoid carrier for 29 days	....	>1:20	27,000 per c c	>30 days
1049	Typhoid infection 19 days; purulent, blood, coagula, etc.	>1:20,000	1:600	14,000,000 per c c	>60 days
1083	Typhoid infection, 7 days; died	1:10,000	1:100	63,000,000 per c c	12th day
1160	Typhoid carrier, 66 days; purulent	1:2000	1:10	11,800,000 per c c	>44 days
1164	Typhoid carrier, 65 days	1:2000	....	317,000,000 per c c	5th day
1491	Typhoid infection 1 day	1:20,000	.. ..	5,000,000 per c c	5th day
			Guinea-Pigs		
322	Typhoid infection 7 days	1:100	....	>1000 per c c	2d day
326	Typhoid infection 21 days; died	....	....	∞ B. typhosus	2d day
			Monkey		
S.I. Macacus rhesus	Carrier for 14 days; light green, thin bile	1:8000+++	1:10	Innumerable	5th day
			Human		
Mrs. Bl.	Cholecystectomy following typhoid fever (2/26/18)	....	....	30,000,000 per c c	20th day incubator dried out; room temp. >79 days; in ice chest >79 days
			Dogs		
3	Intravenous, 24 hours after infection, killed	<1:10	<1:10	30 per c c	Exposed to air 21 days; paraffin oil >30 days
6	Intravenous, 4 hours after infection, killed	<1:10	<1:10	2 per c c	Exposed to air >30 days; paraffin oil >30 days

always viscid and behaved like true gallbladder biles. The only human bile examined was purulent, and registered, after exposure to air, only minimum changes in the reaction, which naturally favored the viability of B. typhosus therein. The draining gallbladder of the patient was found free of typhoid bacilli 28, 36, and 50 days after the specimen for the test-tube experiments was collected, and yet in vitro the organism continued to flourish. We mention this fact merely as a paradoxical curiosity which again illustrates our belief that test-tube experiments are frequently misleading artefacts.

IS THE ANTISEPTIC EFFECT THE RESULT OF BACTERIOLYTIC ANTIBODIES?

The observations of Buxton,[60] Bull,[62] and others recently confirmed by Teague and McWilliams [63] have shown that normal rabbit serum is capable of killing large numbers of typhoid and paratyphoid A bacilli. The close relationship between the blood and the secreted bile in the liver shown by the experiments dealing with the passage of microorganisms from the interlobular veins to the bile capillaries suggested carrying out some tests to determine the presence of bacteriolysins. It was also necessary to settle more satisfactorily the questions dealing with the elimination of agglutinins and antibodies in the bile.

According to Vincent [16] and his pupils, who diligently investigated the presence of complement-fixing antibodies and the so-called "substance anti-sensibilisatrice" in the bile of typhoid immune rabbits, bacteria thrive in the bile of such animals on account of the failure of antibodies to reach the gallbladder or because the antibodies are not persistent on account of the continuous flow and renewal of the secretion. The latter supposition is supported by the observation that the fixing bodies are either absent (in 2 only out of 9 biles examined) or disappear rapidly from the viscus. Forster and Kayser state that bile of immunized rabbits occasionally exhibited bactericidal properties in the peritoneal cavity of guinea-pigs when used in a Pfeiffer bacteriolysin test.

The results of our tests with fresh hepatic duct bile of normal rabbits kept on different diets are given in table 3. We also tested the bile of some immune rabbits and guinea-pigs. With the exception of the latter, which apparently destroyed uniformly 1,000,000 organisms in from 24 to 36 hours, the results were practically identical with those illustrated in the table and are therefore not recorded in detail.

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[62] J. Exper. M., 1915, 22, p. 475.

[63] J. Immunol., 1917, 2, p. 93.

Rabbits' hepatic duct bile is capable of destroying any number of living typhoid bacilli in from 5 to 7 days when kept in test tubes exposed to air. Irrespective of the bacteria inoculated, growth was always present with as small a number as 5 organisms, although it rarely extended over the 48th hour.

There is an absence of a graded diminution in the number of viable organisms as ordinarily seen in quantitative biologic tests for serum bacteriolysis; on the contrary, a sudden destruction of a large number of living typhoid bacilli takes place on the 5th day. The serum of the same rabbit is capable of destroying 1,000,000 bacteria in 1 c c of serum in 24 hours. Bacteriolytic antibodies in an immunologic sense are not active in rabbits' hepatic bile, and other factors probably explain the interesting bactericidal properties of this secretion.

The same conclusion can be applied to the bile of other animals, namely, the antiseptic property is not due to bacteriolysins or "a substance sensibilitrice."

CAN AGGLUTININS BE DEMONSTRATED IN BILE?

In this connection we investigated the readily demonstrable immune bodies in the hepatic duct and cystic bile of normal, immune and infected rabbits and guinea-pigs. The findings of agglutinins in the gallbladder biles of immunized rabbits (also guinea-pigs?) reported by Cantani[11] are contradicted by the observations of Forster and Kayser,[64] Venema[65] and Schöbl on rabbits and by Stäubli,[66] who studied the subject in guinea-pigs. Stäubli pointed out that bile from cadavers collected many hours after death may contain agglutinins and that the collection of bile from highly immunized animals (guinea-pigs) must be conducted with great care in order to avoid the admixture of traces of blood, which may readily produce positive agglutination reactions in the otherwise agglutinin-free bile. The presence of clumped bacteria in the gallbladder content is, however, commented upon by Cushing,[67] Richardson,[68] Doerr[69] and others.

In our tests we followed the customary technic, using formalinized standardized suspensions both for the bile and serum samples.

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[64] München. med. Wchnschr., 1905, 31, p. 1473; 52, p. 1476.

[65] Berl. klin. Wchnschr., 1906, 43, p. 99.

[66] Centralbl. f. Bakteriol., I, O., 1903, 33, p. 375.

[67] Bull. Johns Hopkins Hosp., 1898, 9, p. 91.

[68] J. Boston Soc. of Med. Sc., 1898-99, 3, p. 29.

[69] Centralbl. f. Bakteriol., I, O., 1905, 39, p. 624.

Seven normal rabbits had neither in the hepatic duct nor in the gallbladder bile agglutinins above the dilution of 1:10. The cystic bile obtained by laparotomy from 3 immunized rabbits in a series of 13 tested, agglutinated formalinized typhoid bacilli in a dilution of 1:60 or even 1:100. The remaining 10 agglutinated in a dilution of 1:10 or not at all. Bile derived from 10 rabbits which had been chloroformed in various stages of immunization or recovery from infection in 2 animals gave a reaction as high as 1:200 and 1:1000, respectively. Three recovered carriers with marked thickening of the gallbladder wall agglutinated in a dilution of 1:400-600. One immunized animal, which died from an intercurrent infection, was necropsied 8 to 10 hours after death and furnished a cystic bile which clumped typhoid bacilli in a dilution as high as 1:1000. Six normal and 8 immunized guinea-pigs gave hepatic and cystic duct biles which were free from agglutinins irrespective of the serum agglutination titer that they possessed. Our observations confirm those of Foster and Kayser[64] (p. 1476) and indicate that 25% of the immunized rabbits or recovered carriers may possess agglutinins in the bile. The source of these immune substances cannot be determined with certainty. Some of the rabbits with positive bile specimens had liver injuries and showed the lesions of a chronic cholecystitis or had indications of ascites on account of renal injury caused by a preceding typhoid infection. The degree of the agglutination was entirely independent of the serum agglutinins. The absence of such antibodies in the hepatic duct bile is ample proof, in our opinion, that a true secretion of agglutinins in the same sense as we accept it to be the case for the milk does not take place for the bile, at least in rabbits and guinea-pigs. This fact supports our contention that the antiseptic effect of bile cannot be the result of true, biologically demonstrable antibodies.

A few words on the agglutinins of carrier biles should, however, be added in this connection. An extensive series of gallbladder bile derived from infected animals in various stages of the infection were at our disposal. A total of 31 rabbits was examined; 25 animals were chloroformed, 2 laparotomized and 5 specimens were collected after death. Twenty-five, or 78% of the samples, gave distinct agglutination reactions in dilutions above 1:10, nearly 56% of these biles reacted even in a dilution of 1:100 and above. The strongly positive biles were derived from rabbits, which showed gallbladders in a state of stasis, enormously distended and filled with a pigmentless, slightly purulent bile. The gallbladder wall was often diphtheritic or in old carriers thickened by the inflammatory process. In others the agglutinin content was due to a slight contamination with blood, and in still others the collection many hours after death must be considered responsible for the presence of agglutinins. The number of viable B. typhosus present, not the duration of the carrier stage, apparently influenced the antibody content. The correlation of the findings suggests, that prolonged biliary stasis in the diseased gallbladder due to obstruction of the cystic duct by sandlike carbonate débris or a severe necrotizing, inflammatory process in this viscus are conducive to the presence of agglutinins, and explains the regular finding of clusters of typhoid bacilli in the sediment derived from such biles. How far these antibodies influence the course and duration of the carrier state is difficult to say. As a rule, progressive repair of the inflammatory cholecystitis and hepatitis is shown by a return of the normal color of the bile and is usually accompanied by a marked diminution or even complete disappearance of the agglutinins irrespective of the presence of B. typhosus. Gallbladders with impaired function, irrespective of their agglutinin containing bile, more often remain infected for longer periods than the unobstructed ones.

QUANTITATIVE TESTS TO DETERMINE THE FACTORS RESPONSIBLE FOR THE ANTISEPTIC EFFECT OF HEPATIC DUCT AND GALLBLADDER BILE OF RABBITS, GUINEA-PIGS, DOGS, AND OXEN

From the review of the literature we gained the impression that the reaction, the bile salts, the biliary lecithin and cholesterin play either alone or in combination the rôle of disinfecting substances in bile specimens tested in vitro. The selective action and the variant behavior of the biles of the different animals may in part be the result of the composition of the secretion. This is particularly true with regard to the bile salts. According to Hammarsten[70] human, rabbit, monkey and ox bile contain mostly, and pig bile entirely, the sodium salt of glycocholic acid. On the other hand, in dog, sheep and goat, bile taurocholic compounds either predominate or are found exclusively in many specimens. The antiseptic properties of the various bile salts in different concentrations (a test-tube experiment) will be treated in a separate paper.

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[70] Lehrbuch der physiologischen Chemie., 1914, p. 390. Ergebn. d. Physiologie., 1905, 4, p. 1.

In this connection attention is called to the recent observation of Davis and Hain[71] and to our own, that rabbits' urine possesses a germicidal action, particularly against the organisms of the colon-typhoid group. The antiseptic properties are inconstant in that all urines do not possess equal action nor are all specimens of urine from the same rabbit of like potency. The action is influenced by diet, but is independent of the H-ion concentration of the urine. The nature of the substances responsible for these germicidal properties is as yet unknown. At the beginning of our studies on the antiseptic action of bile we were favorably inclined to suspect an analogy between biliary and urinary antisepsis, but subsequent tests did not support this view. In most of the experiments the urine specimen of rabbits or guinea-pigs, which gave a strongly antiseptic bile, permitted a free development of B. typhosus and allied organisms.

This and other observations stigmatized the reaction as the most important factor in the germicidal action of hepatic duct bile. As early as 1886 Charrin and Rogers[11] came to a similar conclusion and expressed their view in the following sentence (p. 426): "Est-ce à sa reaction que la bile en nature doit ce pouvoir antiseptique moindre que celui de quelques unes de ses élements." Nichols[1] was even more convinced that the alkalinity of the hepatic duct bile of rabbits and guinea-pigs was responsible for its germicidal action because the latter disappeared on neutralization. The importance of the reaction was also recognized by Meyerstein[54] in his tests with various mixtures of bile salts; a neutral or slightly acid reaction favored the growth of B. typhosus, while an alkaline reaction was decidedly inhibitive.

In the early period of the research on immunity, von Fodor,[72] Emmerich[73] and others advanced the theory, based on observations made on rabbits infected with anthrax, that the bactericidal properties of the serum are closely connected with the alkalinity of the blood. The influence of the reaction is undoubtedly only secondary and plays only a subordinate rôle. This theory has therefore been entirely disregarded. The preliminary bile studies, which have given the desired information to conduct the quantitative tests reported below, are presented in the preceding papers. Having established the optimum H-ion concentration for the growth of B. typhosus and also its generation time, we studied in detail the changes which take place in the hepatic duct bile of rabbits and guinea-pigs on standing exposed to air or alkali. It was found that on account of the escape of CO_2, and possibly the absorption of ammonia, the H-ion concentration decreased progressively in 24 hours to a P_H^+ of from 8.6 to >9.0; in a similar manner the titrable alkalinity increased to a marked degree. On the other hand, it was demonstrated that these changes could be retarded by the stratification of the biliary effluent with paraffin oil during the act of collection and during the experiments. We have stated in this connection that the reaction of hepatic duct bile in vitro exposed to air for even a short time differs materially from that present in the animal body. Similar changes in the reaction to a less marked degree occurred in the cystic bile and a final P_H^+ over 8.4 was occasionally recorded. It was also pointed out that the diet and the biliary stasis provoked by starvation affect the H-ion concentration of the gallbladder bile.

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[71] J. Urolog., 1918, 2, p. 309.

[72] Arch. Hyg., 1886, 4, p. 129.

[73] Emmerich, E., and Wagner, G.: Centralbl. f. allg. u. path. Anat., 1916, 27, p. 433. Ztschr. f. Immunitätsforsch. u. exper. Therap., 1916, 24, p. 557.

These principles were applied to the study of the antiseptic effect of hepatic duct bile of rabbits and later extended to that of guinea-pigs, oxen and man. Definite amounts (usually 5 c c, cystic bile 1-2 c c) of fresh bile collected under oil, together with broth controls of a definite P_H^+, were seeded with 300-500 typhoid or dysentery bacilli or Vibrio cholerae and kept in a water bath at 37 C. At varying time intervals 0.1 c c amounts were plated either directly or in progressive dilutions in peptic digest agar. After 24-36 hours' incubation at 37 C. the plates were counted. It is customary to use the logarithm of the number of viable organisms in recording results of experiments on the rate of growth of bacteria. In the accompanying charts the logarithms of the viable organisms per 0.1 c c are plotted as ordinates against time intervals as abscissae. Bile samples, which show by plating active, vigorous growth, are never turbid; they may when exposed to air change their color to an olive-brown, but when stratified with oil they always remained smaragadine green. It was also noted that certain types of bacteria reduced the biliary pigment more readily than others.

From an extensive series of identical tests we select a few illustrative examples.

THE RATE OF GROWTH AND THE SUBSEQUENT DISINFECTION OF RABBIT BILE

Chart 1 illustrates the rate of growth of B. typhosus in the hepatic bile of a rabbit kept on an oat diet, compared with the one in buffered salt free broth. Similar experiments conducted with hepatic duct bile of rabbits kept on a mixed and on a cabbage diet gave identical results. Hepatic bile of rabbits, when tested in vitro exposed to air and alkali favors in the first 24 hours the growth of B. typhosus; later it acquires germicidal properties, which lead, irrespective of the diet of animals, to complete disinfection of this body fluid in 96 hours. This definite cycle of events, so well illustrated graphically, can be prevented by stratification of the hepatic duct bile with oil. Cessation of growth occurs in the stratified bile at the same time as in the broth, and the disappearance of viable typhoid bacilli progresses in a similar manner. As the typhoid bacilli inoculated in the bile specimens were grown in a broth of a reaction of P_H^+ 7.0, transplantations to mediums of more alkaline reactions (P_H^+ 8.4-8.6) were expected to produce a decided "lag."

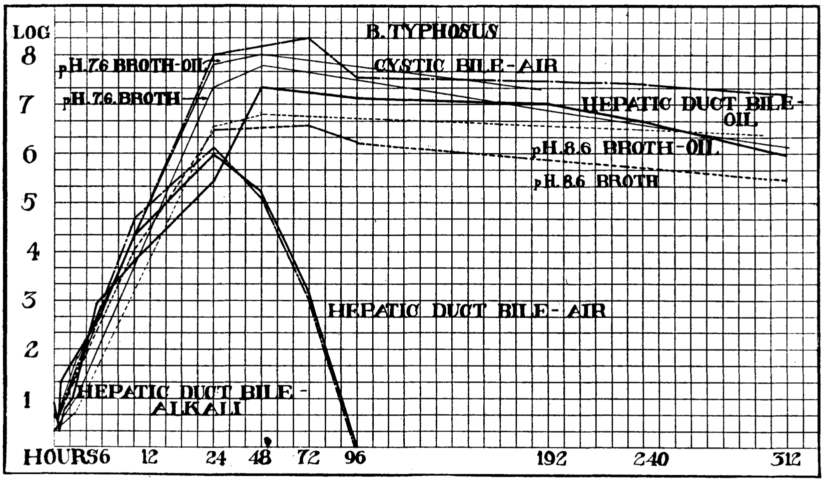

Chart 1.—Rate of growth of B. typhosus in the hepatic duct bile of a rabbit kept on an oat diet compared with growth of the bacillus in buffered salt-free broth.

If we assume for the present that the nutritive requirements are satisfactory for the growth of B. typhosus and recall that this organism has a distinct adaptability to changes in H-ion concentration; although it is more alkalo—than acidophilic, it appears not at all surprising that the initial growth in all the bile specimens is good. Once accustomed to the environment, the organisms continue to grow slightly or remain viable even when the reaction has undoubtedly reached an unfavorable point, above P_H^+ 8.8. The acquired alkali tolerance of B. typhosus in milk illustrates this well. Test-tube experiments with a salt-free broth containing 1% of peptone with phosphate mixtures and sodium hydroxide have shown that the "alkali death point" for B. typhosus (K) in such a medium varies between P_H^+ 9.2 and 9.4. A substratum containing more peptones or colloids pushes the "death point" to P_H^+ 9.6 or even 9.8.

When bile is exposed to alkali, progressive decrease in the H-ion concentration following the escape of CO_2 may reach its lowest level in from 10 to 12 hours at 37 C.; yet the typhoid bacillus continues to grow only to be inhibited and destroyed 36 to 48 hours later. Other organisms may develop in the same environment for periods extending over 10 days. This phenomenon was called to our attention by the experiment illustrated in chart 2. The alkalophilic Vibrio cholerae grows favorably in rabbit bile covered by oil and is completely killed in the secretion exposed to air after an incubation time of 12 days. Cessation of growth takes place in all tubes inclusive of the broth control after 48 hours' incubation. The progressive disappearance of viable organisms proceeds slowly in the broth and bile tube stratified with oil, and more rapidly in the tubes exposed to air or alkali. The decrease in the tube kept in the desiccator is apparently interrupted after the 144th hour. In this experiment the tube remained unopened in the desiccator for 6 days and was found to contain a large number of viable vibrios on the 12th day of incubation. The reaction of this specimen

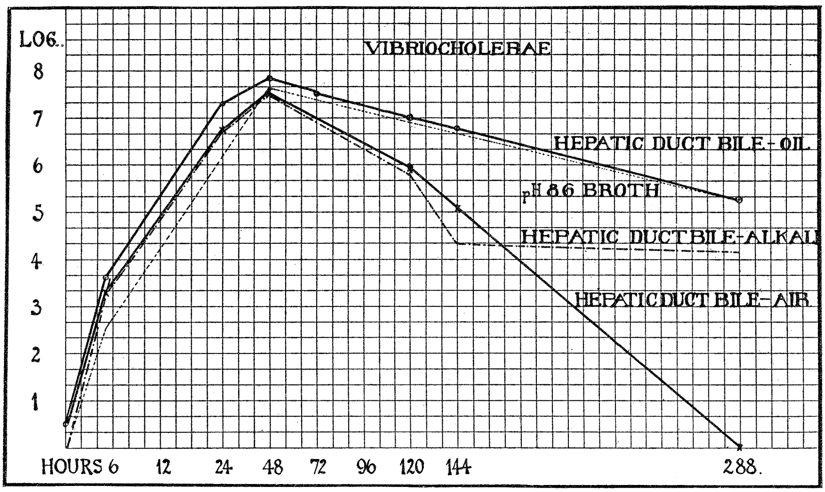

Chart 2.—Rate of growth of Vibrio cholerae in the hepatic duct of a rabbit.

differed only slightly from that exposed to air, and yet the spirillae remained viable. The absence of germicidal properties for Vibrio cholerae in bile kept in a sealed desiccator is rather difficult to explain. The nature of the factors operative on the bile in this environment, in contrast to that exposed to air, is not clearly understood. Three possibilities suggest themselves: (1) absorption of water and concentration of the bile (accurate quantitative tests were not made but casual observations failed to support this particular contention); (2) continuous absorption of the ammonia produced by the growth of the organisms; and (3) the prevention of ammonia absorption from the air.

Old bile exposed to the air at room temperature or in the ice-chest is antiseptic the first 24 hours for B. typhosus and B. dysenteriae Shiga. This observation explains the results of Nichols and our own in the qualitative series. Rabbit hepatic duct bile collected in open tubes and kept at room temperature even for 24 hours will exhibit strongly bactericidal properties; the inoculated bacteria are unable to grow and are progressively destroyed after 15 hours of incubation. Such biles can be partially rejuvenated by saturation with CO_2 and subsequent stratification with oil. Various bacteria are enabled to grow at least for 15 hours but subsequently germicidal substances make their appearance and sterilize the hepatic bile in 48 hours when exposed to air, or in from 192 to 216 hours when stratified with oil.

A similar cycle of events takes place in bile samples which are neutralized by the addition of HCl or lactic acid. These experiments were merely undertaken to verify the observations of Nichols.[1] It is quite obvious that the addition of strong acids, such as hydrochloric and sulphuric as recommended by this worker, may, aside from neutralizing the carbonates, materially change the chemical composition of the secretion and produce conditions which never occur in the animal body. Neutralization of fresh and old bile, which is germicidal in 24 to 96 hours, produces a hepatic duct bile suitable for the growth of a variety of bacteria. Invariably the antiseptic properties manifest themselves in the neutralized biles in from 1 to 8 days later than in the untreated ones. Bile specimens which are neutral show a decline in the H-ion concentration probably due to an escape of CO_2 or to an absorption of ammonia. The inoculated types of bacteria, however, grow well and remain viable for over 10 days. Such biles are antiseptic for Vibrio cholerae. The change in the H-ion concentration is therefore in all probability only one of the many factors which is altered under the influence of the neutralization process.

THE GERMICIDAL PROPERTIES OF HEPATIC DUCT BILE IN EXPERIMENTAL ALKALOSIS AND ACIDOSIS

In the preceding paper we discussed the experiments which were undertaken with the intention of determining the H-ion concentration of hepatic duct bile under the influence of an experimentally produced alkalosis or acidosis. It was stated that the reaction of this secretion is rarely changed on account of the feeding or the injection of $NaHCO_3$ or HCl. It was, however, shown that the reaction of the cystic bile of rabbits may be distinctly influenced by the same treatment with these chemicals or by fasting. In the discussion of the qualitative tests reported in the preceding paragraphs of this paper, it was shown that acid-forming diets may produce a hepatic duct bile which becomes only germicidal after 96 to 168 hours' standing at room temperature. These facts naturally suggest some experiments in order to prove or to refute the contention of Nichols that an alkaline therapy may be of value in preventing and curing typhoid gallbladder carriers. Animals kept on acid-forming or base-forming diet were in the course of the experiment treated with alkalies, and the hepatic duct bile collected before and after the administration of the chemicals was submitted to qualitative tests.

The intravenous injection of sodium bicarbonate produced in one rabbit a hepatic duct bile in which B. typhosus was killed. From another rabbit a similarly active bile specimen was collected after the administration of 2 gm. of $NaCO_3$ intravenously and of 5 gm. by stomach tube. An animal kept on a base-forming diet for 16 days and intensively alkalinized before the operation furnished a hepatic duct bile and a cystic bile which differed in no respect from those obtained from rabbits kept on a base-forming diet alone. These few experiments are suggestive, but further tests on a large series of animals are necessary before final conclusions can be reached. It is quite obvious from the observations already reported that we are dealing in those germicidal tests with test-tube experiments, and that we have no knowledge of the physiologic or pharmacologic action of alkalies on the rabbit organs and tissues or on the composition of the secreted bile. Observations to be reported in a subsequent paper indicate that alkalinization of typhoid infected rabbits may act indirectly in preventing the accompanying acidosis and not through their immediate action on the organisms themselves. It is in this sense that we feel the experiments of Nichols may finally be interpreted, when all the factors concerned have been fully analyzed.

TABLE 3

BACTERIOLYTIC POWER (?) OF HEPATIC DUCT BILE OF RABBIT FOR TYPHOID BACILLI

Rabbit	Diet	Dosage of Bacilli per C c of Bile	Progress of Sterilization Days							Remarks
			1	2	3	4	5	6	7	
1418	Mixed	710,000	+	+	+	+	0	..	..	Open in tube with cotton stopper
Serum agglut:	diet	300,000	+	+	+	+	0	..	..	One c c of serum destroyed
1:5		20,000	+	+	+	+	0	..	..	1,000,000 in 24 hours
Bile agglut:		1,500	+	+	+	+	0			
<1:5		184	+	+	+	0				
		23	+	+	0					
		12	+	+	0					
		6	+	+	0					
		3	+	0						
1418	Mixed	160	+	+	+	+	+	+	+	Under paraffin oil
Serum agglut:	diet	23	+	+	+	+	+	+	+	
1:5		12	+	+	+	+	+	+	+	
Bile agglut:		2	+	+	+	+	+	+	+	Growth on 35th day
<1:5										
1181	Oats	500,000	+	+	+	+	0	..	..	Open tube; cotton stopper
		250,000	+	+	+	+	0			
		25,000	+	+	+	+	0			
		2,500	+	+	+	+	0			
		500	+	+	+	+	0			
		50	+	+	+	+	0			
		25	+	+	+	+	0			
		12	+	+	+	+	0			
		5	+	0						
1182	Cabbage	847,000	+	+	+	+	+	0	..	Open tube, cotton stopper
		423,500	+	+	+	+	+	0		
		42,350	+	+	+	+	+	0		
		4,235	+	+	+	+	+	0		
		847	+	+	0					
		84	+	0						
1200	Cabbage	500,000	+	+	+	+	+	0	..	Open tube, cotton stopper
	and	250,000	+	+	+	+	+	0		
	$NaHCO_3$	25,000	+	+	+	+	+	0		
		2,500	+	+	+	+	+	0		
		500	+	+	+	+	+	0		
		50	+	+	+	+	+	0		
		25	+	+	+	+	+	0		
		12	+	+	+	+	0			
		5	+	+	0					

As the germicidal action of hepatic duct bile is probably closely connected with the available bases in this secretion, an investigation of the behavior of this fluid obtained from an animal impoverished of these substances was important. A state of acidosis is readily produced in rabbits by feeding hydrochloric acid. The experiments were similar to those reported in the foregoing. The acid was fed to the rabbits operated on after the collection of the necessary control samples.

With the exception of rabbit 1031 b, no appreciable influence on the germicidal properties of hepatic duct bile of rabbits in a state of experimental acidosis could be noted. From this animal, after a total feeding of 0.72 gm. of HCl, a bile sample was obtained which sterilized itself of 2 strains of B. typhosus after 192 instead of 144 hours. The viability of B. dysenteriae was prolonged for an additional 24 hours. The minor differences recorded for the bile specimens of rabbits 32 and 1118 may be safely attributed to the method of testing the progress of sterilization and not to any material change in the bile itself. It is possible that more definite results would have been obtained if the animals had been kept on acid-forming diets and if the treatment with HCl could have been extended over a longer period. For practical purposes a continuous mild acidosis can probably be more satisfactorily obtained by fasting or by a predominant oat diet.

TABLE 4

THE PROGRESS OF STERILIZATION OF HEPATIC DUCT BILE OF RABBITS DILUTED WITH DISTILLD WATER

Four Rabbits. Diet: Mixed oats and carrots.

Changes Approximately Produced in Bile	PH	Fistula Bile: 1:1000 to 1:10,000 Loopful of a 24-Hour Broth Culture								
		Typhoid K	Typhoid C	Typhoid J	Typhoid B	Paratyphoid A	B. Dysenteriae Shiga	Paradysenteriae Strain	B. coli	V. cholcrae
1 c c bile	8.4	72	84	96	72	96	24	24	132	228
1 part bile + 1 part distilled water	..	150	192	192	192	216	24	24	288	408
1 part bile + 2 parts distilled water	..	264	276	204	360	336	48	60	384	408
1 part bile + 3 parts distilled water	..	336	288	276	324	360	48	96	384	408
1 part bile + 9 parts distilled water	..	264	312	312	408	384	48	156	408	408
1 c.c distilled water	..	24	24	24	24	24	24	36	132	72
1 c c peptone (0.1%) solution	..	400	408	408	408	408	108	240	408	408

DILUTION AND THE ADDITION OF SERUM TO HEPATIC DUCT BILE OF RABBITS

Observations reported above suggest that the germicidal properties of hepatic duct bile in all probability depend on other factors than merely progressively increasing alkalinity. Among the factors to be considered are the lack of nutritive material and the influence of H-ion concentration on the action of the bile salts. The first problem was investigated by progressively diluting bile samples or by adding heated sterile rabbit serum to strongly bactericidal bile specimens and inoculating each specimen with small amounts of typhoid bacilli.

In table 4 we summarized our results obtained on 4 hepatic duct bile samples collected from animals kept on various diets. It is quite evident that the progress of sterilization is retarded for from 3 to 5 days, when the bile specimen is diluted with distilled water in the proportion of 1:1. Moreover, progressive dilution produces a pronounced retardation in the antiseptic effect of one and the same bile sample. The distilled water used was doubly distilled and free from CO_2. B. typhosus and most of the other bacteria refuse to grow in it. These observations clearly demonstrate that the antiseptic properties of hepatic duct bile of the rabbit cannot be the result of a lack of nutritive substances. The H-ion concentration of the secretion exposed to air is only indirectly responsible for the gradual destruction of the inoculated micro-organisms. Even in a poorly buffered medium, such as the bile diluted 1:1 or even 1:2, the recordable change in the H-ion concentration is negligible and for practical purposes has no influence on the viability and growth of the inoculated bacteria. The actual hydrogen-ion concentration is decreased in a dilution of 1:9, and yet the various typhoid strains remained viable for more than 10 days in comparison to the short period of 4 days noted in the control tubes. It is our belief that dilution reduces the main germicidal substances, probably the effect of the bile salts. The time rate of lethal action is prolonged, irrespective of the low, unfavorable H-ion concentration. It is possible that the procedure of diluting bile induces other alterations, which we have not as yet investigated carefully. Even a profound change in the protein and salt content appears to be more favorable for the persistence of viable B. typhosus than the concentrated secretion supposedly rich in nutritive material.

In this connection it was naturally of interest to see how far the addition of nutritive material, for example serum, influences the rate of lethal action. According to Pies [23] and others, bile becomes regularly a good culture medium by the addition of organic matter, such as blood serum, pus or aleuronate. In table 5 the average time rate of lethal action of 8 hepatic duct biles diluted with serum on 2 strains of B. typhosus and 1 paradysentery strain is compared with that of undiluted and diluted bile. Contrary to the general belief expressed in the literature, fistula bile fortified by the addition of serum is not a medium in which B. typhosus can develop indefinitely. For example, the destruction in a specimen diluted with the serum in the proportion of 1:1 or 1:2 is identical with the one obtained for the same specimen diluted with distilled water. The prolongation of the viability of the organisms is therefore purely the result of a dilution of the antiseptic substances and not due to the addition of nutritive material or "vitamines." This conclusion is supported by the figures given in table 5; as has already been shown, the addition of small amounts of serum had no influence in its prolific effect. Our data support the conclusion of Pies that dilution with saline solution does not exert the same influence as distilled water. Further experiments are necessary before we feel justified in offering an explanation for this interesting observation.

The next factor or group of factors to be analyzed would be the bile acids and their disinfecting properties at various H-ion concentrations. Certain observations made on dog bile containing an excess of bile salts were so suggestive that we considered it advisable to conduct an extensive series of quantitative tests which will be reported in a separate paper.

TABLE 5

THE PROGRESS OF STERILIZATION OF HEPATIC BILE DILUTED WITH HEATED RABBIT SERUM

Rabbit	Diet	Amount of Heated Serum Added to Fresh Bile	P_{H^+}	Fistula Bile		
				Typhoid Kl 172	Typhoid K 120	Para-dysentery 310
8 Rabbits	Mixed Oats Cabbage and Carrots	1 c c bile	8.1	124	110	36
		1 c c serum	..	>240	>240	..
		1 c c bile + 1 c c serum	..	216	192	..
		1 c c bile + 0.5 c c serum	..	192	168	96
		1 c c bile + 0.25 c c serum	..	204	168	..
		1 c c bile + 0.2 c c serum	..	114	...	36
		1 c c bile + 0.1 c c serum	..	104	90	12
		1 c c bile + 0.05 c c serum	..	68	84	..
		0.95 c c bile + 0.05 c c serum	..	168	...	..
		0.75 c c bile + 0.25 c c serum	..	192	216	..
		0.5 c c bile + 0.5 c c serum	..	266	266	..
		1 c c bile + 0.5 c c saline	..	96	96	..
		1 c c bile + 0.5 c c distilled water	..	168	168	..
		0.5 c c bile + 0.5 c c distilled water	..	216	216	..

THE PROGRESSIVE STERILIZATION IN BILE DERIVED FROM HYPERCHOLESTEROLIZED RABBITS

Among the chemical components of the bile the various lipoids, soaps and cholesterol fractions deserve some consideration in the light of recent observations. Manfredi,[57] for example, reports that a concentration of cholesterin of 0.5% added to culture mediums will check typhoid and the paratyphoids, while B. coli will grow in concentrations up to 1%. It is worth noting that Bacmeister[74] and Exner and Heyrovsky[75] observed the decomposing effect of bacteria of the typhoid-paratyphoid group on bile, which frequently resulted in the precipitation of cholesterin. The experiments of Bassenge [55] with lecithins also deserve consideration. This worker demonstrated that these substances in 1% emulsions are destructive for typhoid bacilli in from 30 to 60 minutes. On the other hand, the growth-stimulating properties of unheated lecithin-containing mediums for B. anthracis and the tubercle bacillus is well known. Moreover, we recall the studies of Morato and Villanueva,[45] who demonstrated that cholesterol injections hasten the production of antibodies and agglutinins.

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[74] München. med. Wchnschr., 1908, pp. 211, 283 and 339.

[75] Arch. f. klin. Chir., 1908, 86, p. 609.

In order to determine the germicidal properties of lipoids and cholesterol we conducted a small series of tests with hepatic duct bile derived from hypercholesterolized rabbits. A fairly marked hypercholesterolemia was readily produced in these animals by feeding either lanolin (Merck's C. P.) or sheep brain or egg yolks. In the preceding paper data were recorded which demonstrated conclusively that it was possible to increase in rabbits the demonstrable blood cholesterol. Such a condition frequently led to a distinct precipitation of this substance in the gallbladder bile. The hepatic duct bile reached on one occasion only a concentration of 0.1% cholesterol, which according to the findings of Manfredi [57] is insufficient to exert a checking influence on B. typhosus. On the other hand, the gallbladder bile gave a concentration of 0.5%, despite which the growth and persistence of the viable B. typhosus were unhampered. The biles of cholesterolized rabbits were not any more germicidal than those of normal rabbits kept on various diets. Tests not detailed in this paper have confirmed in a general way these observations, and have shown that biles derived from fasting animals, which invariably have an increased cholesterol content (Rothschild [76]), differ little in their germicidal properties from those of normal animals.

Next in order for investigation would be the biliary mucin and the surface tension. A study of the latter is contemplated. An investigation of the antiseptic properties of biliary mucin appeared superfluous. Hepatic duct bile, which becomes antiseptic, is free from this substance, and cystic bile, which occasionally contains a considerable amount, is a good medium. This justifies the impression that the admixture of this secretion to hepatic duct bile of rabbits is in part responsible for the absence of germicidal properties.

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[76] Beitr. z. path. Anat. u. z. allg. Path., 1915, 66, p. 227.

A number of experiments were made by using ether or chloroform extracts of hepatic duct bile of rabbits. No difference in the influence on the growth of B. typhosus was observed in such biles in contrast to those untreated.

THE GROWTH OF B. TYPHOSUS IN HEPATIC DUCT BILE ENCLOSED IN COLLODIUM SACS IN THE PERITONEAL CAVITY OF THE RABBIT

It has been shown that hepatic duct bile of rabbit removed from the body becomes antiseptic when exposed to air. In an effort to rejuvenate the old antiseptic bile we returned it to the animal body in collodium sacs. This transformation of germicidal bile into a good medium was apparently accomplished in the following experiment.

Exper. 1.—A paralodium sac prepared over a glass guide with 4 holes sterilized in live steam for 1 hour was filled with 4 c c of hepatic duct bile (reaction P_H^+ 8.4) of rabbit 1182. This bile when tested immediately and when exposed to air destroyed 100 organisms per c c in 48 to 72 hours. The bile in the paralodium sac was seeded with an average of 50 typhoid bacilli (Strain K). The sac was carefully sealed and transferred to the peritoneal cavity of rabbit 1190. After 6 days had elapsed this animal was killed and carefully necropsied and cultures made. The tissues were found free from typhoid bacilli. The paralodium sac was covered by a thin layer of fibrin: there was no leakage of the membrane. The contents were pale greenish with a slightly brownish tinge and were perfectly clear. One c c contained 2.5 million viable B. typhosus. The reaction was P_H^+ 6.7.

It is evident that old, antiseptic hepatic duct bile in a paralodium sac, seeded with a small number of B. typhosus becomes a good culture medium for these organisms when placed in the peritoneal cavity of a rabbit. The most obvious change noted in this bile was an increase in the H-ion concentration; practically the optimum for B. typhosus had been attained. Naturally, this is only one of the changes which probably took place, and it would be erroneous to conclude that we reproduced in this experiment the conditions as they exist in the gallbladder. The observation is, however, of considerable interest, and further experimentation, particularly with longer exposure to the peritoneal fluid, may furnish important data on the influence of bile on B. typhosus and similar questions discussed in this paper.

THE RATE OF GROWTH AND THE SUBSEQUENT DISINFECTION IN GUINEA-PIG BILE

A series of quantitative tests with the hepatic bile of guinea-pigs that had been collected under oil, conducted in an identical manner to those reported for the rabbit, demonstrated, when seeded with B. typhosus, B. dysenteriae and Vibrio cholerae, a similar change in the rate of growth and in the subsequent development of antiseptic properties. One set of experiments is chosen in charts 3, 4, and 5, to illustrate the points of importance.

In contrast to our qualitative tests with bile collected unprotected in open tubes, in which most of the selected bacteria were destroyed in 48 to 96 hours, guinea-pig hepatic duct bile collected under oil is a good culture medium for the first 24 to 48 hours of incubation, and develops after this period germicidal properties for B. typhosus and B. dysenteriae. Bile samples kept exposed to the air or in a desiccator become antiseptic sooner for Vibrio cholerae than for B. typhosus and B. dysenteriae. It is, furthermore, shown that the same bile stratified with oil preserves the growth of Vibrio cholerae and also of B. typhosus, but does not prevent the development of germicidal substances for B. dysenteriae. The disappearance of viable dysentery bacilli occurs simultaneously in the tubes exposed to air or to alkali, or in the desiccator, as it does in the tubes stratified with oil. This event is fairly constant for a number of hepatic duct biles studied, and represents a noteworthy difference from the rabbit biles. It appears rather unlikely that under these conditions the development of germicidal properties is directly connected with a change in the reaction.

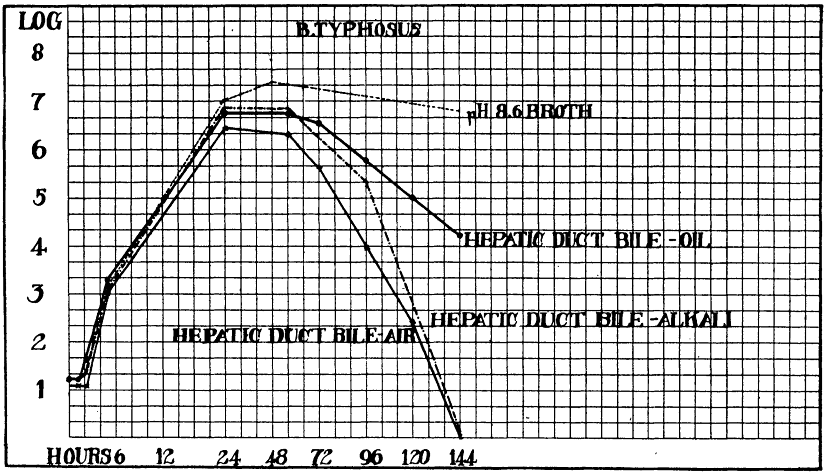

Chart 3.—Rate of growth of B. typhosus in the hepatic duct bile of guinea-pigs.

This suspicion is supported to a certain degree by the findings made on Vibrio cholerae. Chart 5 demonstrates the disappearance of viable spirilla in the tubes exposed to air and in the desiccator on the 96th and 144th hour, respectively. It is recalled that these organisms remain viable in rabbit bile for at least 288 hours, and in the desiccator even longer, at a period of the experiment when the alkalinity has probably reached its maximum (P_H^+ 9.2; titrable — 0.75). The persistence of bacteria rather sensitive to alkalies, like the Shiga dysentery organism in contrast to the alkalophilic Vibrio cholerae which disappears rapidly from guinea-pig bile, suggests the coexistence of other factors more potent than the change in the H-ion concentration as the agents responsible for the germicidal properties of hepatic duct bile exposed to air. Such substances develop rapidly on standing, when bile is collected in open tubes.

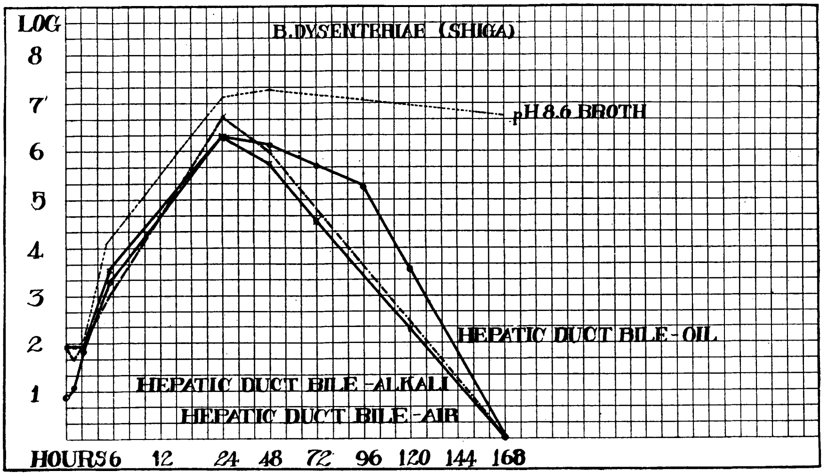

Chart 4.—Rate of growth of B. dysenteriae (Shiga) in the hepatic duct bile of guinea-pigs.

In another experiment bile was collected in open tubes and exposed to air for 4 hours before inoculation with B. typhosus. At the same time as a control a sample was stratified with paraffin oil. The sample exposed to air favored the growth for 5 to 8 hours; then germicidal substances produced a progressive disappearance of viable organisms. In 48 hours, sometimes in 24 to 36 hours, such a bile sample was completely sterilized. This experiment explains fully the results of Nichols, who collected the bile in open tubes and kept it at room temperature for more than 3 hours.

It has been repeatedly stated that dilution has little effect on the H-ion concentrations of bile. The tests under consideration are in every respect identical to those reported for rabbit bile with the exception that guinea-pig bile diluted with distilled water produces a more favorable medium than when diluted with saline. The distinct reciprocal relationship between dilution and viability of B. typhosus is also clearly demonstrated. The greater the dilution, the longer the persistence of viable organisms. Old and decidedly bactericidal biles, which destroy large numbers of B. typhosus in 6 hours, favored the growth and viability of these bacteria even in dilutions of 1 :1 or 1 :2. Differences in the H-ion concentration could not be recorded by our crude colorimetric method. The addition of 0.25 c c of sterile, heated guinea-pig serum prolonged the existence of viable bacteria in the proportion to the resulting dilution and not as would be expected in accordance with addition of nutritive material and buffers. Irrespective of all the changes brought about by these manipulations, the hepatic bile of guinea-pigs acquired antiseptic properties after a time which depended entirely on the degree of the dilution.

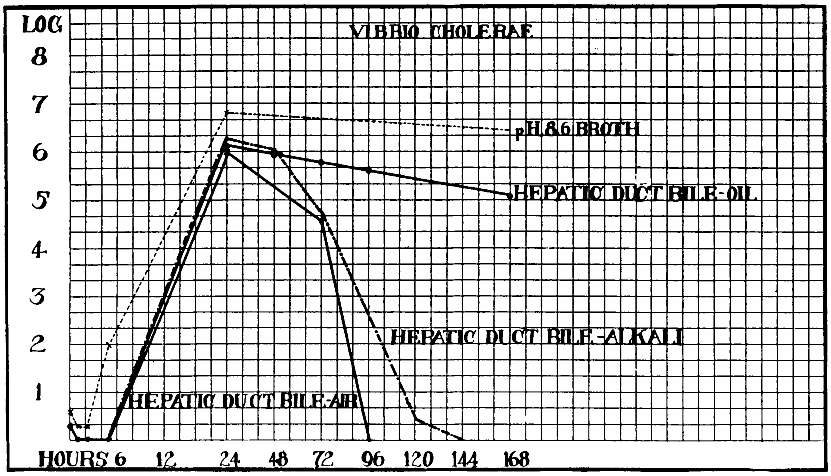

Chart 5.—Rate of growth of Vibrio cholerae in the hepatic duct bile of guinea-pigs.

Neutralization with lactic or metaphosphoric acid prolonged the persistence of viable B. typhosus for a few days. The results of these experiments were practically identical to those reported for the rabbit and the same interpretation can be applied to the guinea-pig's hepatic duct bile.

THE RATE OF GROWTH AND THE SUBSEQUENT DISINFECTION IN DOG BILE

The rate of growth and the lethal action of hepatic duct and of cystic bile procured from two dogs is shown in chart 6. Both animals showed a marked reflex in the secretion of the duct bile, and received either sodium taurocholate or ox bile during or before the collection of the bile specimens. Dog 17-170 secreted a nongermicidal bile for B. typhosus before the injection of sodium taurocholate. The pooled specimens collected after the injection were decidedly antiseptic as is clearly indicated by the curve in chart 6. A large number of typhoid bacilli was destroyed in less than 60 hours. Dog 17-220 was fed ox bile 4 hours previous to the operation and the collection of the bile specimens. B. typhosus grew fairly well for 30 to 40 hours. The bile, however, acquired antiseptic properties and after 120 hours was found to be sterile.

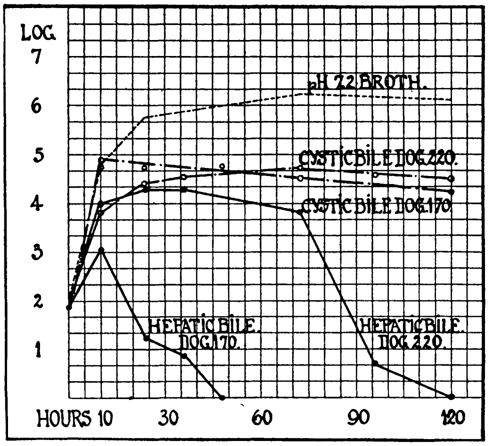

Chart 6.—Rate of growth in and lethal action of hepatic duct and cystic bile procured from two dogs.

Our observations which confirmed Ecker's,[19] prove the inhibitive and germicidal properties of bile salts on B. typhosus. In the course of test-tube experiments with bile salts added to suitable mediums of varying H-ion concentrations, it was noted that a P_H^+ over 8.0 markedly increased the bacteriostatic properties of bile salts in concentrations of less than 0.5%. A 1% solution of sodium taurocholate in a weak peptone salt solution at a P_H^+ of 8.4 was strictly germicidal. These observations have a direct bearing on the explanation of the germicidal properties of the two hepatic duct biles collected from dogs which were treated with sodium taurocholate in order to overcome the reflex inhibition of the bile flow. The familiar cholagogue action of sodium taurocholate given intravenously or by mouth, and the fact that from 40 to 90% of the introduced bile salts are eliminated in 4 to 6 hours (Foster, Hooper and Whipple [77]), immediately suggest a relationship between the increased amount of bile acids and the germicidal properties of hepatic duct bile. The absence of an antiseptic action of cystic bile is explained by the fact that the method of collecting the bile prevented the entrance of hepatic duct bile into this receptacle. These conclusions were confirmed by a quantitative analysis of the bile acids present in the hepatic duct bile of dog 17-170 and dog 17-220. We gratefully acknowledge the assistance received from the late Miss M. G. Foster, who made the determinations of taurocholic acid by the method developed by herself. The elimination of taurocholic acids were:

Dog 17-170 (weight 14.5 lbs.): Before the intravenous injection of 1 gm. of sodium taurocholate the hepatic duct bile contained 8 mg. of taurocholic acid per c c. In this sample B. typhosus grew for more than 10 days. After the intravenous inoculation of 2 gm. of bile salts the pooled bile specimen collected for a period of 5 hours (total 28.8 c c), contained 103 mg. per c c (or five times the maximum amount ordinarily found in hepatic duct bile or 1% of sodium taurocholate).

Dog 17-220 (Weight 45.25 lbs.): This dog was fed 400 c c of fresh ox bile by stomach tube 3 hours preceding the operation and the collection of bile samples. The pooled hepatic bile collected for a period of 4½ hours (total 106.9 c c) contained 63 mg. taurocholic acid or over 0.005% of sodium taurocholate.

The bile samples collected from the two dogs possessed, therefore, abnormal amounts of bile acids. In combination with the progressively increasing H-ion concentration, a strongly germicidal medium was produced. The gradual disappearance of viable bacteria in these bile samples is therefore readily explained. The absence of antiseptic properties in hepatic duct bile of some dogs treated with sodium taurocholate was due to a high H-ion concentration or perhaps to an absence of the cholagogue action of the administered bile salts or to both. It is obvious that the explanation offered may not be the only one, and that further investigations alone can furnish the answer to some of the questions which remain unsolved in this preliminary inquiry.

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[77] J. Biol. Chem., 1919, 38, pp. 379, 354.

THE RATE OF GROWTH AND THE SELECTIVE ANTISEPTIC ACTION IN FRESH AND OLD CYSTIC BILE OF THE OX

Fresh, unheated cystic bile of the ox inoculated with a large amount of typhoid bacilli still contained, after 10 days' incubation, viable bacteria. Fornet,[20] Ecker,[19] Pies [23] and others found certain bile antiseptic properties. Quantitative tests were made with fresh unsterilized gallbladder biles collected from beef animals in San Francisco during the months of March and April.

According to chart 7, comparatively small amounts of B. typhosus (strain K) in fresh unheated ox bile when stratified with oil grew freely, perhaps not as rapidly as in broth of a P_H^+ of 8.6. Even in the desiccator the development and persistence of viable organisms was fair. Decidedly antiseptic were the same bile specimens (5 c c) when kept exposed to ordinary laboratory air. A 24-hour period of active growth was followed by a progressive disappearance of living B. typhosus, and sterility was usually attained between the 72nd and 120th hour. The colorimetric titration gave always P_H^+ readings >8.6. The reaction changed slowly from a slightly, to a strongly, alkaline one, and regularly reached a lower H-ion concentration than the specimens kept in the desiccator, in which the absorption of ammonia was prevented. On the other hand, samples covered with oil showed occasion-

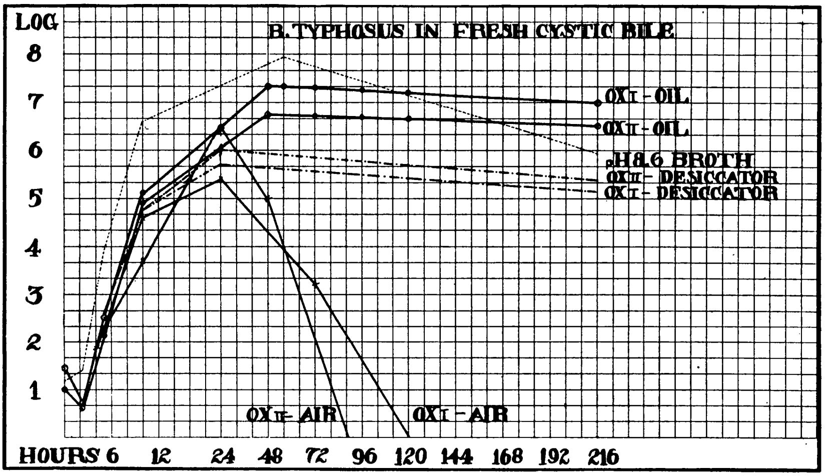

Chart 7.—Rate of growth of B. typhosus in fresh cystic bile of oxen.

ally a slight increase in the H-ion concentration from P_H^+ 8.0 to P_H^+ 7.6, probably due to the liberation of CO_2 by the cells or to lactic acid formation, the result of bacterial fermentation or destruction of cellular elements on standing. The difference in the behavior of ox bile in comparison to rabbit's and guinea-pig's bile was found in the tubes kept in a desiccator. We are inclined to explain this difference on the basis of a higher H-ion concentration. Furthermore, hepatic duct bile of rabbits has a different composition than cystic bile of oxen, and changes in the H-ion concentration may produce more potent germicidal forces in the former than in the latter. The varying composition of cystic ox bile intimately connected with the food of the animal is of greatest importance. It explains, in all probability, the varying results reported by different investigators.

The selective action of fresh unheated ox bile on Vibrio cholerae and B. dysenteriae Shiga was also studied. According to Schöbl, ox bile is a good culture medium for Vibrio cholerae. There may be a slight inhibitive action and the indication of a lag. The growth may be less heavy than in a plain salt-free peptone broth of a P_H^+ of 8.6. B. dysenteriae, however, cannot thrive in ox bile exposed to air; invariably 1500 organisms or less were destroyed in from 12 to 48 hours. This corresponds with the findings of Marbais.[78] Some specimens stratified with oil sterilized themselves of B. dysenteriae in 168 hours. In all three samples in which this took place, a shift of the H-ion concentration above P_H^+ 8.4 was noted and reached in the light of the observation of Cohen and Clark [79] the border of the P_H^+ zones. Reproduction and viability of B. dysenteriae Shiga is completely impaired. Tests with Flexner bacilli produced similar results. Occasionally, bile specimens were found which permitted an indefinite growth of these organisms, as Flu [12] has recorded for beef biles freely exposed to air.

Ox bile kept at room temperature exposed to diffuse light or in the ice-chest under oil or exposed to air and heated at 60 degrees for 30 minutes or autoclaved, produced a medium with a peculiar action on B. typhosus, which is illustrated by chart 8. In the experiments on which this chart was based, 5 c c samples of bile were inoculated with about 500 living B. typhosus. Antiseptic properties reduced this number materially in from 1 to 10 hours. This period of destruction of viable organisms varied with the different bile samples. The degree of germicidal action was occasionally so marked that a 0.1 c c specimen was found to be free from viable B. typhosus. If a small number of bacteria was seeded in such biles, a mortality of 100% or a complete sterilization might ensue, confirming Fornet [20] and Pies.[23] As a rule, however, the germicidal action on the part of the medium was followed by a pronounced more or less varying "lag" which in turn was succeeded by a gradual multiplication of the apparently seriously injured cells. Progressive proliferation of B. typhosus extended over 7 days, and not only reached, but sometimes surpassed the total number of viable organisms present in the broth control. This characteristic behavior of old heated cystic bile of the ox as a medium for B. typhosus is apparently not connected with the H-ion concentration of the secretion. Not only were the specimens acid to litmus and phenolphthalein, but the P_H^+ never decreased below 8.2. In the course of the experiment, probably as a result of the growth of B. typhosus, it even increased to P_H^+ 8.0 or even 7.6. From the standpoint of reaction, the ox bile became therefore more and more suitable. It is not unlikely that simultaneously a new strain was gradually generated with an acquired resistance to the antiseptic and bacteriostatic properties of the old bile. This new strain may in part be responsible for the slow but progressive

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[78] Compt. rend. Soc. Biol., 1918, 81, p. 1136.

[79] J. Bacteriol., 1919, 4, p. 409.

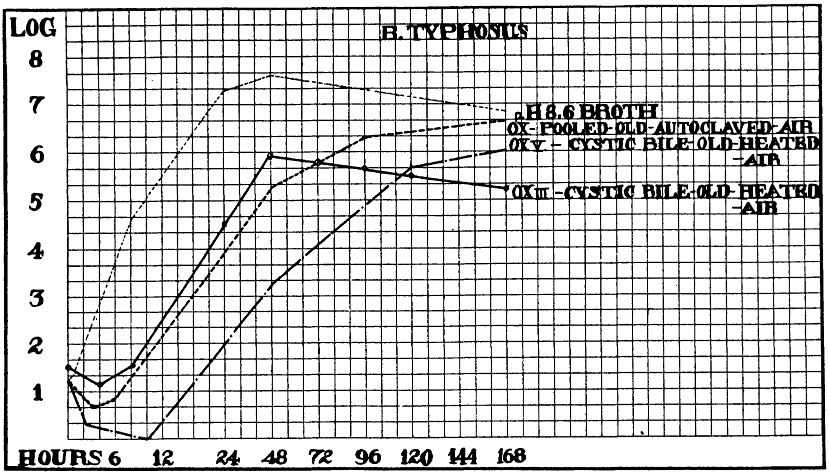

Chart 8.—Rate of growth of B. typhosus in bile of an ox.

proliferation after the 24th hour. A limited number of observations made by successive transfers of B. typhosus through biles of the same animal support this explanation.

As repeatedly emphasized, our study of ox bile was primarily undertaken to correlate the findings made with biles of other laboratory animals and to demonstrate conclusively that any bile on standing in a test tube undergoes changes which produce a germicidal, sometimes even a selective action, on a variety of bacterial types. Heating, filtering and other manipulations only enhance these properties and produce finally in vitro a medium of such complexity that it is impossible even to surmise how it acted in its natural environment, the gallbladder. The recognition of this fact, which does not apply to the study of bile alone but to many other body secretions, is frequently ignored by the majority of bacteriologists.

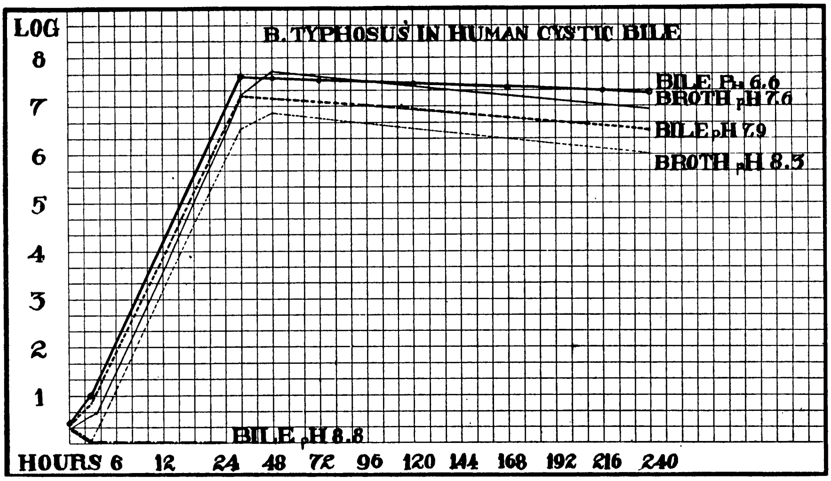

Chart 9.—Rate of growth of B. typhosus in human cystic bile.

THE RATE OF GROWTH OF B. TYPHOSUS AND VIBRIO CHOLERAE IN HUMAN CYSTIC BILE AT VARYING H-ION CONCENTRATIONS

Human cystic bile collected at necropsy or during laparotomies or cholecystectomies is, as a rule, a good medium for the development of a variety of bacteria which can remain viable for 60 days and longer. (Toida [27] and others.) Hepatic fistula bile proves a good medium for every micro-organism inoculated. Some investigators, Pies,[23] Mieczkowski,[32] Hirokowa,[30] and Toida [27] report, however, a marked degree of initial inhibition for a number of samples studied. We were unable to find such a specimen and can therefore not express an opinion on the nature of this bacteriostatic action. It is rather remote to suspect a change of reaction to be also responsible for this "lag," as was the case for the various other biles investigated. Our observations have demonstrated that the fairly alkaline or neutral human cystic bile changes slightly when standing exposed to air.

Fresh human bile was tested to compare its behavior with that in which the H-ion concentration had been previously artificially changed. The growth curves are shown in charts 9 and 10.

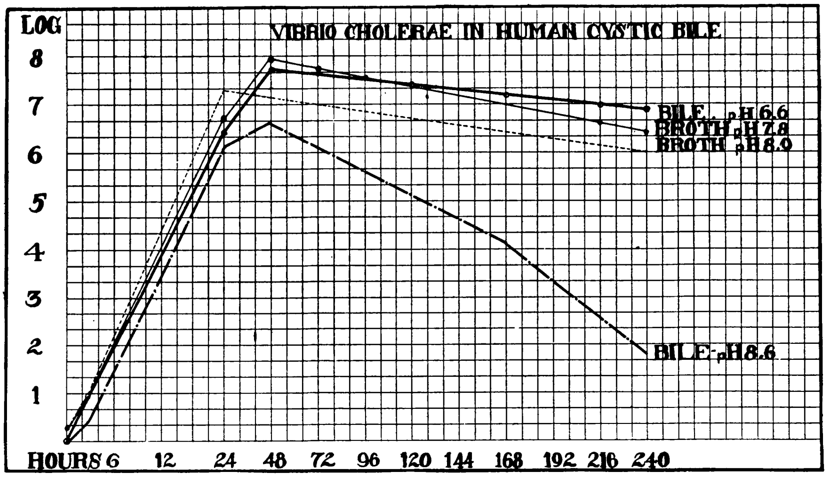

Chart 10.—Rate of growth of Vibrio cholerae in human cystic bile.

It is evident that the proliferation of B. typhosus and Vibrio cholerae is only impaired or even completely suppressed at a H-ion concentration which in all probability never occurs in the human gallbladder. Human cystic bile must be considered a suitable medium for the viability of the two organisms; at least, in comparison with ox or rabbit bile the vigor of the bacterial growth is noteworthy. The sample chosen for the determination of the growth curves shown in the charts was derived from a gallbladder with a slight stasis as a result of fasting. The growth-favoring influence of starvation on the hepatic duct and cystic bile of rabbits has been previously mentioned. It was also pointed out that stasis favors gallbladder infection and therefore the carrier state. The growth curves presented here may therefore be of practical significance and suggest a possible connection between suitability of cystic bile as a medium for B. typhosus and for Vibrio cholerae; this may also explain the gallbladder carrier state. Variability in the composition, for example, changes in the proportion of taurocholic to glycocholic acid, and perhaps the reaction, influence the selective growth of different bile samples, as is well illustrated by the observations of Hirokowa,[30] Toida,[27] and others; but only a large series of tests will demonstrate beyond a doubt the actual importance and influence of the bile as such in the human typhoid carrier state.

SUMMARY

Hepatic duct bile of rabbits collected in open tubes and seeded with various pathogenic and nonpathogenic intestinal bacteria exhibits selective germicidal properties. The organisms of the typhoid-dysentery-paratyphoid group remain viable for 72 to 96 to 120 hours, and those of Vibrio cholerae and staphylococcus for over 7 to 10 days. A small number of bacteria is more readily destroyed than those of a heavy inoculum. Base-forming diets produce, on the average, bile specimens in which the time rate of lethal action is slightly shorter than in those procured from animals fed on acid-forming diets. True gallbladder bile specimens collected from the same animals and tested under the same experimental conditions fail to exhibit germicidal properties in the chosen time interval of 10 days.

Guinea-pigs' hepatic duct and cystic bile exhibits under identical experimental conditions greater germicidal action against the same number and the same type of bacteria than rabbit-liver bile. B. typhosus, B. paratyphosus, and B. dysenteriae are usually destroyed in from 24 to 48 hours; even Vibrio cholerae cannot survive a period of 120 hours. Fresh cystic bile of guinea-pigs, accidentally suffocated, was not germicidal for 400 hours.

Gallbladder bile of dogs kept on a mixed diet is apparently indifferent for a variety of bacteria; the inoculated organisms remain viable for over 10 days. Hepatic duct bile of the same animals treated with bile salts as cholagogues may be germicidal and destroy B. typhosus in from 24 to 120 hours.

Qualitatively at least the hepatic duct bile of cats, goats, rats, monkeys and man, as well as the gallbladder bile of oxen, sheep and pigs

favor the viability of B. typhosus. In such bile specimens inoculated with a large number of bacteria, living germs could be demonstrated in open tubes after 10 days of incubation at 37 C. Typhoid bacilli show a high degree of viability in human hepatic duct and cystic bile. Vibrio cholerae may be destroyed in 8 days. We failed to observe in one case of human biliary fistula after the institution of an extensive alkaline therapy, the development of germicidal properties against B. typhosus.

Nonpurulent gallbladder biles of typhoid-infected or carrier rabbits, guinea-pigs, monkeys and man behave either like hepatic duct bile specimens or like true cystic bile samples when removed from the viscus and exposed to air. Purulent specimens from rabbit or man, when incubated at 37 C., may show viable bacteria for over one month. The physical consistency, the cellular contents, and the duration of the carrier state govern the persistence of living B. typhosus in such specimens.

The germicidal properties of hepatic duct bile of rabbits and of guinea-pigs is not due to bacteriolysins or a "substance sensibilatrice." Such specimens never contain agglutinins. According to our observations, about 25% of the gallbladder bile specimens derived from laparotomized immune rabbits may contain agglutinins in a dilution of over 1:50. Recovered carriers may give clumping in dilutions above 1:100. The biles of guinea-pigs are always found to be free from agglutinins. One-half of the biliary secretions collected from "carrier" rabbits agglutinate B. typhosus in dilutions above 1:100. The degree of the inflammatory process and the stasis in the gallbladder are probably some of the factors which are conducive to the presence of agglutinins.

Quantitative tests with hepatic duct bile of rabbits collected under oil but tested in vitro exposed to air or to alkali have shown that B. typhosus grows, following a short lag, for at least 24 hours. However, in the next 24 to 48 hours, the bile acquires, probably on account of changes in the H-ion concentration (escape of CO_2, absorption of ammonia), antiseptic properties which lead, irrespective of the diet of the animal, to complete disinfection of the fluid in 96 hours. In hepatic duct bile stratified with paraffin oil and in cystic bile B. typhosus shows a high degree of viability. The Vibrio cholerae behaves similarly. These facts disprove, in our opinion, the theory that rabbit bile is germicidal in vivo.

Old, sterile bile kept at room temperature or in the ice-chest is germicidal for B. typhosus and B. dysenteriae Shiga after 24 hours' exposure at 37 C. Such bile specimens can be rejuvenated by saturation with CO_2 and subsequent stratification with oil. Neutralization of germicidal bile may produce temporarily, at least, a fluid in which a variety of bacteria can develop for from 1 to 8 days. The addition of strong acids alters the bile in many respects. Feeding or the injections of alkalies and acids, which produce a state of experimental alkalosis or acidosis, do not materially influence the germicidal properties of the hepatic duct bile of rabbits.

The H-ion concentration of the hepatic duct bile is only indirectly responsible for its antiseptic properties. Dilution of this secretion with distilled water in the proportion of 1:1 or even 1:2 fails to change the reaction but alters the concentration of the bile salts. It prolongs the persistence of viable bacteria for several days in comparison to the undiluted control tubes. Sterile serum acts in the same manner as distilled water. The growth-enhancing influence is in all probability not caused by the addition of nutritive material or "vitamines." The hepatic duct bile of hypercholesterolized rabbits develops germicidal properties after the same time interval as was observed for normal rabbits.

Antiseptic rabbit bile sealed in collodion sacs and placed in the peritoneal cavity of rabbits was found to be an excellent medium for the growth of B. typhosus.

Fistula bile of guinea-pigs collected under oil is a good medium for B. typhosus, Vibrio cholerae and B. dysenteriae. On standing exposed to air it changes its reaction and acquires germicidal properties which destroy the viable organisms in from 96 to 168 hours after inoculation and incubation at 37 C. One strain of an alkalophilic Vibrio cholerae was apparently more readily destroyed than several strains of B. typhosus and B. dysenteriae. The initial "lag" in fresh bile is slight and of short duration. Bile collected in open tubes or exposed for 4 to 24 hours to air becomes germicidal in a shorter time interval than fresh bile. The inoculated bacteria are destroyed in 24 to 48 hours or even less. Such biles, when diluted with distilled water or saline, favor the persistence of viable B. typhosus irrespective of the H-ion concentration. A distinct reciprocal relationship exists between dilution and viability of bacteria. B. typhosus will remain viable for over 10 days when the hepatic duct bile of the guinea-pig is diluted in the proportion of 1:9.

Fistula bile collected from dogs which received ox bile by mouth or sodium taurocholate intravenously in order to counteract the post-operative reflex inhibition on the biliary system, may contain more than 0.005% of taurocholic acid per c c. The salts of this acid are strongly antiseptic in an alkaline medium. As the hepatic duct bile of the dog also becomes alkaline (P_H^+ 8.2-8.4) on standing exposed to air, it is not surprising to observe the development of germicidal properties for B. typhosus in such specimens.

Fresh unheated sterile cystic ox bile develops, on incubation and exposure to air, germicidal properties. A certain number of the viable B. typhosus and B. dysenteriae are prevented from multiplication, and a large number of bacteria is actually destroyed in the first two hours after transference to the ox bile medium. This period is followed by a "lag" of varying length, which in turn is succeeded by a rapid proliferation. The maximum development is reached on the 12th or 48th hour for the specimen exposed to air. Progressive destruction, however, sterilizes these specimens in from 12 to 120 hours. The time required depends entirely on the bile specimen. The Vibrio cholerae is less seriously injured than typhoid and dysentery bacilli. Sterilization of the samples exposed to air or alkali has not been noted. Ox bile prevented by stratification with oil from giving off CO_2 or from absorbing ammonia in a desiccator does not become germicidal. Old, heated or unheated ox bile acts in a characteristic manner on B. typhosus. Immediately following the inoculation of the bile a marked destruction of viable organisms is recorded. This germicidal action may lead to complete sterilization of the bile, or is followed by a pronounced lag, which in turn is succeeded by a slow, but progressive, multiplication of the bacteria. The maximum growth may only be attained after 7 days, and the growth curve may at this time not only reach but even surpass the one of the broth control. This action of the bile is entirely independent of the reaction of the secretion. Our experiments confirm those of other workers, and they should prove conclusively that cooked ox bile is unsuitable for the primary isolation of B. typhosus from tissues or secretions. Mixed with peptone and glycerol it is, on account of its hemolytic properties, a good substratum for typhoid blood cultures.

The vigor of the bacterial growth in an average human cystic bile is noteworthy. Proliferation can be influenced by artificially reducing the H-ion concentration. Complete suspension of growth, however, only occurs at a P_H^+ which in all probability never occurs in the human body. The study of a large series of human cystic biles is advocated to determine the actual importance and influence of the secretion as such on the human typhoid carrier state. The observations reported in this paper have in our opinion demonstrated one outstanding fact, namely, a test-tube experiment may be simple and reveal a great deal of information, but it does not always tell the truth and may be most misleading to the pathologist.